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Čedomir N. Radenović[1, 2], *Miloš V. Beljanski*[3], *Georgij V. Maksimov*[4], *Aleksandar A. Kalauzi*[5], *Milan D. Dražić*[6]

[1] Maize Research Institute, Zemun Polje, Biophysics Laboratory, Belgrade, Serbia and Montenegro
[2] Faculty of Physical Chemistry, University of Belgrade, Belgrade, Serbia and Montenegro
[3] Institute of General and Physical Chemistry, University of Belgrade, Belgrade, Serbia and Montenegro
[4] Lomonosov State University 11899 Moscow, Russia
[5] Multidisciplinary Study Centre, University of Belgrade, Belgrade, Serbia and Montenegro
[6] Faculty of Mathematics, University of Belgrade, Belgrade, Serbia and Montenegro

THE MECHANISM OF THE NH_4 ION OSCILLATORY TRANSPORT ACROSS THE EXCITABLE CELL MEMBRANE

ABSTRACT: This paper presents results on typical oscillations of the membrane potential induced by the excitation of the cell membrane by different concentrations of the NH_4Cl solution. The existence of four classes of oscillations of the membrane potential and several different single and local impulses rhythmically occurring were determined. It is known that the oscillatory processes of the membrane potential are in direct dependence on oscillatory transport processes of NH_4 and Cl ions across the excitable cell membrane. A hypothesis on a possible mechanism of oscillatory transport processes of NH_4 and Cl ions across the excitable cell membrane is also presented.

KEY WORDS: plant cell membrane, excited state, membrane potential, oscillatory transport, NH_4 ion

INTRODUCTION

It is generally known that movements, phenomena and processes occurring oscillatory and rhythmically can be found within almost all fields of physics, chemistry and biology. Oscillations are such movements and processes at which, magnitudes of physical quantities determining them, occur at exactly or approximately equal periods of time. Hence, any system disturbed from equilibrium starts to oscillate under certain conditions. Systems with one

or two degrees of freedom are the basis for the analysis of oscillations. Regardless of different types of oscillatory processes, each oscillatory system can be described by a physical quantity whose displacement from its equilibratory value depends on coordinates and time.

The unique mathematical model is used in description of all oscillatory processes, but, homogenous differential equations of the second order with constant coefficients are mainly applied for discrete systems, while partial differential equations with variable coordinates and time are applied for continuous systems (Andronov et al., 1966, Crawford, 1984; Tihonov et al., 1972).

Oscillations and rhythms are one of the principal characteristics of living organisms. A rhythm, as a type of the regularity and autoregulation, with a patterned increase and decrease of certain parameters in the curse of time, has been detected at all levels of organization: molecules, cells, tissues, organs, organisms and the population (Bjunning, 1964, Bioteux et al., 1977).

In recent times the occurrence of oscillations of the membrane potential has been more systematically studied and analysed and therefore it is a very actual and contemporary scientific topic (Koljs et al., 1993, Krainski et al., 1981, Žabotinskij, 1974).

Furthermore, overall bioelectric studies on plant models contributed to a discovery of the phenomenon of the membrane oscillatory potential (Vorobljev et al., 1967, 1968, Radenović, 1974). The actual and genuine phenomenon of the membrane oscillatory potential on the excitable cell membrane (plasmalemma and tonoplast) of intact plant cells was discovered at the end of 1960s (Radenović et al., 1968, Volkov et al., 1968, Vučinić et al., 1973, 1987). The excitation of the plant cell and its membranes was performed under the influence of selected factors (concentration, mechanical, temperature, luminous, etc.) under which the oscillations of the membrane potential are generated (Radenović and Penčić, 1970, Radenović and Vučinić, 1987, Vuletić et al., 1987). To that effect, the same or similar is related to the membrane oscillatory potential induced by concentrations of monovalent cations (Radenović et al., 1977, Radenović and Ratković, 1982). There are no integral papers on the membrane oscillatory potential studied at different NH_4 ion concentrations in the available literature. In this paper we report, for the first time, a more detail investigation of the membrane oscillatory potential induced by different NH_4 ion concentrations, the same as the explanation of oscillatory transmembrane transport across the excitable cell membrane.

MATERIALS AND METHODS

The bioelectrical experiments for producing oscillations of the membrane potential at different NH_4 ion concentrations were performed on living cells of the fresh-water alga *Nittela*. Growing conditions and the standard preparation of *Nitelle* cells for bioelectric measurements had already been described in our

studies (Radenović et al., 1968, Radenović and Penčić, 1970, Radenović, 1974).

All chemicals were of the pa. grade, prepared fresh prior to experiments.

Single and local impulses and complete oscillations of the membrane potential were registered after the method with a microelectrode technique, which was also previously described, in principle and details, (Radenović et al., 1968; Radenović and Penčić, 1970, Radenović et al., 1976, 1977).

RESULTS

1. *Obtained results of bioelectric standard measurements prior to recording of oscillations of the membrane potential induced by the NH_4Cl solution*

Living cells of *Nittela* grown on the 1% nutrient agar (0.1 mM KH_2PO_4, 1.0 mM $NaHCO_3$, 0.4 mM $CaCl_2$ $6H_2O$ and 0.2 mM $Mg(NO_3)_2$ x $6H_2O$) prior to recording of oscillations of the membrane potential were stabilised by the standard solution for 60 min. Ordinary measurements (membrane stationary potential, cyclosis) were performed by using the standard solution (Table 1) as an external solution. Once the values of bioelectric parameters were within the limits of the normal physiological state of a stabilised living cell, oscillations of the membrane potential can be induced and recorded at different NH_4Cl solution concentrations.

Tab. 1: Bioelectrical parameters of the cell state of alga *Nittela* prior to inducement of oscillations of the membrane potential with the solution with NH_4Cl.

Membrane stationary level (Ψ_m, mV)	Cyclosis (μsec^{-1})	Standard solution for initial bioelectrical measurements
Standard levels:		
—90	45	
—120	50	0.1 mM HCl + 1.0 mM NaCl
—150	52	

2. *Oscillations of the membrane potential induced by different NH_4Cl solution concentrations*

When the standard solution was exchanged for the NH_4Cl solution in the external solution, the excitement of *Nittela* was made and oscillations of the membrane potential were possible to be induced and registered in plasmalemma and tonoplast. The results of 11 typical oscillations of the membrane potential induced by different NH_4Cl concentrations are presented. The stated oscillations can be grouped into four classes and as such shall be presented in this paper:

2.1. *The first class of oscillations of the membrane potential induced by the lowest NH_4Cl concentration (1 mM)*

Figure 1 and Table 2 present the results of oscillations of the membrane potential induced by the solution of the lowest concentration (1 mM NH_4Cl). This typical oscillation is characterised by a pre-oscillatory period with the occurrence of only six single impulses. The possible local impulses or failed oscillations occurred soon after the second single impulse. The irregularity became more pronounced in further observations of oscillations. The end of oscillating passed into chaos.

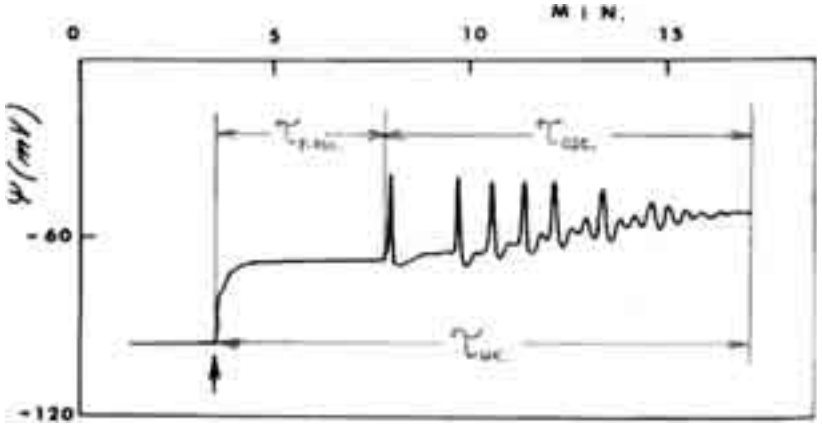

Fig. 1: First class of oscillations. Abbreviations: x — axis: time (min), y — axis: electric potential (mV), arrow start of oscillatory process, τ_{uk}: total duration of oscillations, $\tau_{p.osc.}$: duration of preoscillatory period, $\tau_{osc.}$: duration of oscillatory period. For parameters see Tab. 2

Tab. 2: First class of membrane potential oscillations

Conc. (mM)	Duration of membrane potential oscillation (min)	Duration of preoscillatory period (min)	Duration of oscillatory period (min)	Frequence of singular oscilation appearance (osc/min)	Relative amplitude of oscillation (mV)
1	13.64	4.36	9.28	0.37	27.5 19.0

2.2. *The second class of oscillations of the membrane potential induced by low NH_4Cl concentrations (3—7 mM)*

The results of oscillations of the membrane potential are presented in Figures 2 (A-D), while their characteristics are encompassed by Table 3. Oscillating induced by the solution of 3 mM NH_4Cl (Figure 2 A, Table 3 A) is a somewhat different than the previous one. It had two impulses in the process of depolarisation and seven in the process of repolarisation of equilibration. Possible local impulses could have been observed among single impulses. Oscillating ended in a monotonous stationary state, Figure 2 A, Table 3 A.

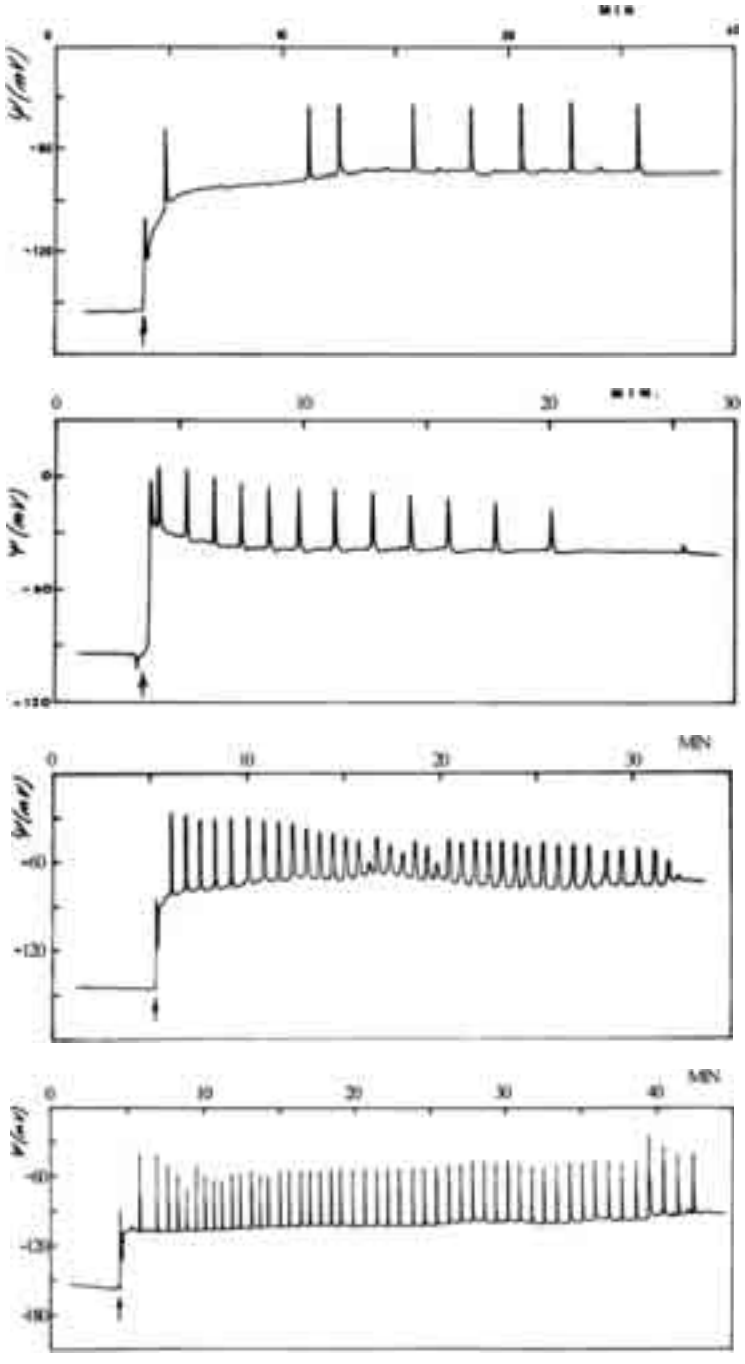

Fig. 2: Second class of oscillations. A: NH_4 ion concentration: 3 mM, B: NH_4 ion concentration: 5 mM, C: NH_4 ion concentration: 6 mM, D: NH_4 ion concentration: 7 mM. For parameters see Tab. 3

Tab. 3: Second class of membrane potential oscillations

	Conc. (mM)	Duration of membrane potential oscillation (min)	Duration of preoscillatory period (min)	Duration of oscillatory period (min)	Frequence of singular oscilation appearance (osc/min)	Relative amplitude of oscillation (mV)	Segment of oscillogram
A	3	25.6	0.09	25.51	0.35	40.35	—
B	5	23.49	0.31	23.18	0.55	30.42	—
C	6	28.28	0.03	28.25	1.27		whole
						56.75↓ 25.38	I
						X_{av} = 21.69	II
						X_{av} = 27.28	III
D	7	38.04	0.00	38.04	1.31	X_{av} = 49.67	—

Oscillating presented in Figure 2 B and Table 3 B (5 mM NH_4Cl) is very similar to oscillating in Figure 2 A. A clearly equilibrated stationary level is observed under depolarisation conditions. Only single impulses with declining amplitudes occurred in this oscillating that ended in an unsuccessful single impulse or a weak local impulse, Figure 2 B, Table III B.

Oscillating presented in Figure 2 C and Table 3 C (6 mM NH_4Cl) acquired characteristics of long-lasting oscillations with a greater number of single impulses (in the processes of depolarisation and repolarisation) up to the stationary level. Three segments can be observed in Figure 2 C. The first oscillation segment started with the single impulse of the highest amplitude and continued with relative damping. The second oscillation segment was bordered with two local impulses within which there were five single impulses of different amplitudes. The third oscillation segment was characterised by the occurrence of a local impulse, Figure 2 C, Table 3 C.

Oscillating presented in Figure 2 D and Table 3 D (7 mM NH_4Cl) also acquired characteristics of long-lasting oscillations with higher NH_4Cl concentrations. Indications of local impulses occurred in the beginning and at the end of oscillating. Oscillating was characterised by alternating and gradual ascending and descending of single impulse amplitudes. A middle part of oscillating (from the 16^{th} to the 46^{th} single impulse) was characterised by consistent amplitudes. At the end of oscillating (the 47^{th} single impulse), amplitudes of single impulses significantly increased, and then gradually decreased till the end of oscillating, Figure 2 D, Table 3 D.

2.3. *The third class of oscillations of the membrane potential induced by NH_4Cl concentrations of 8, 9 and 10 mM*

The results of oscillations of the membrane potential are presented in Figures 3 A-C, while their characteristics are encompassed by Tables 4 A-C.

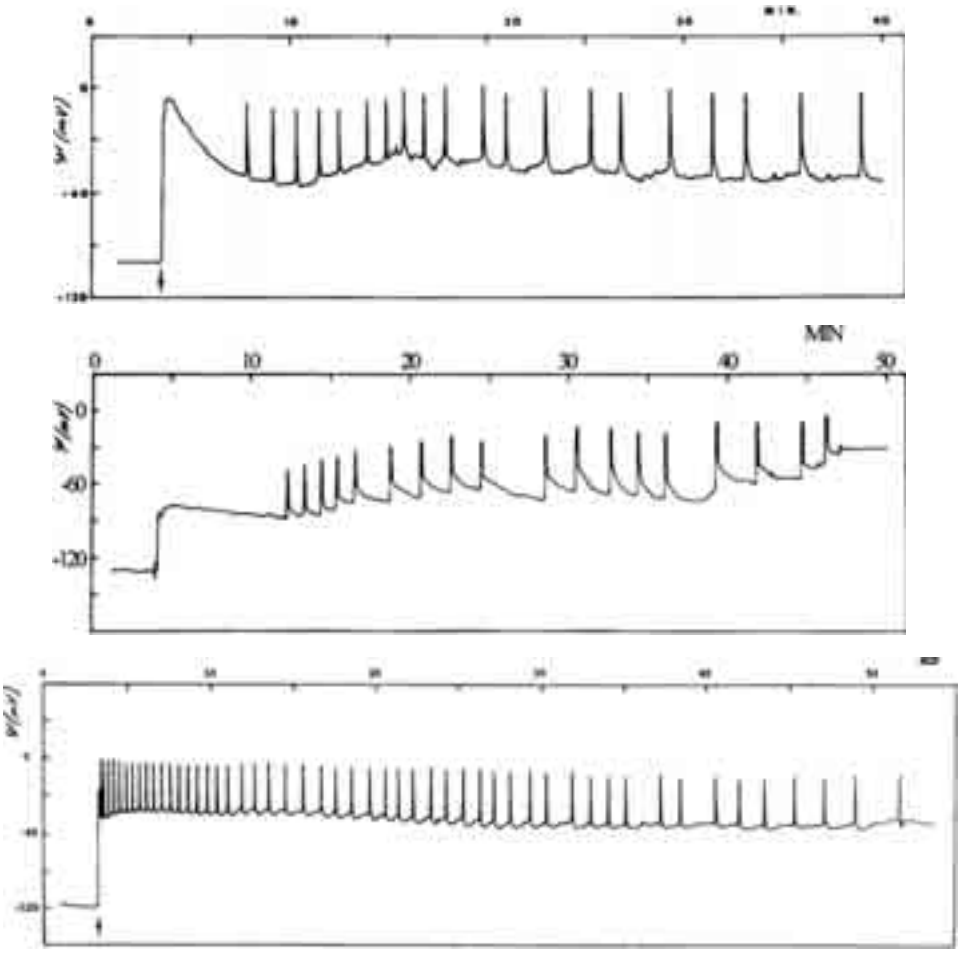

Fig. 3: Third class of oscillations. A: NH_4 ion concentration: 8 mM, B:NH_4 ion concentration: 9 mM, C: NH_4 ion concentration 10 mM. For parameters see Tab. 4

Tab. 4: Third class of membrane potential oscillations

	Conc. (mM)	Duration of membrane potential oscillation (min)	Duration of preoscillatory period (min)	Duration of oscillatory period (min)	Frequence of singular oscilation appearance (osc/min)	Relative amplitude of oscillation (mV)
A	8	35.42	4.26	31.16	0.56	X_{av} = 41.60
B	9	43.15	8.25	34.90	0.42	X_{av} = 44.7
C	10	50.72	0.21	50.51	1.03	X_{av} = 39.02

Since oscillations presented in Figure 3 A and Table 3 A (8 mM NH_4Cl) did not have an equal level of the stationary state, amplitudes of single impulses differed. Local impulses were pronounced during oscillating.

Oscillating presented in Figure 3 B and Table 4 B (9 mM NH_4Cl) was characterised by a pre-oscillatory period. The process of depolarisation lasted till the end of oscillating and was accompanied by single impulses with different amplitudes.

Oscillating presented in Figure 3 C and Table 4 (10 mM NH_4Cl) had characteristics of equilibrated and long-lasting oscillations with a higher number of single impulses and regular processes of depolarisation and repolarisation. However, in comparison with the fourth class of oscillations the duration of this oscillations was not long. The beginning of oscillating was accelerated, but the frequency of the occurrence of single impulses decreased with further spreading of oscillations. An indication of a local impulse occurred almost after every single impulse, Figure 3 C, Table 4 C.

2.4. *The fourth class of oscillations of the membrane potential induced by the highest NH_4Cl concentrations (10 mM)*

The results of oscillations of the membrane potential are presented in Figures 4 A-C, while their characteristics are encompassed by Table 5 A-C.

Oscillating presented in Figure 4 A and Table 5 A (10 mM NH_4Cl) had a pre-oscillatory period in which the indication of local impulses occurred. Besides, this oscillating in the process of depolarisation had the indication of single impulses occurrence, similar to the process of gradual repolarisation. Recor-

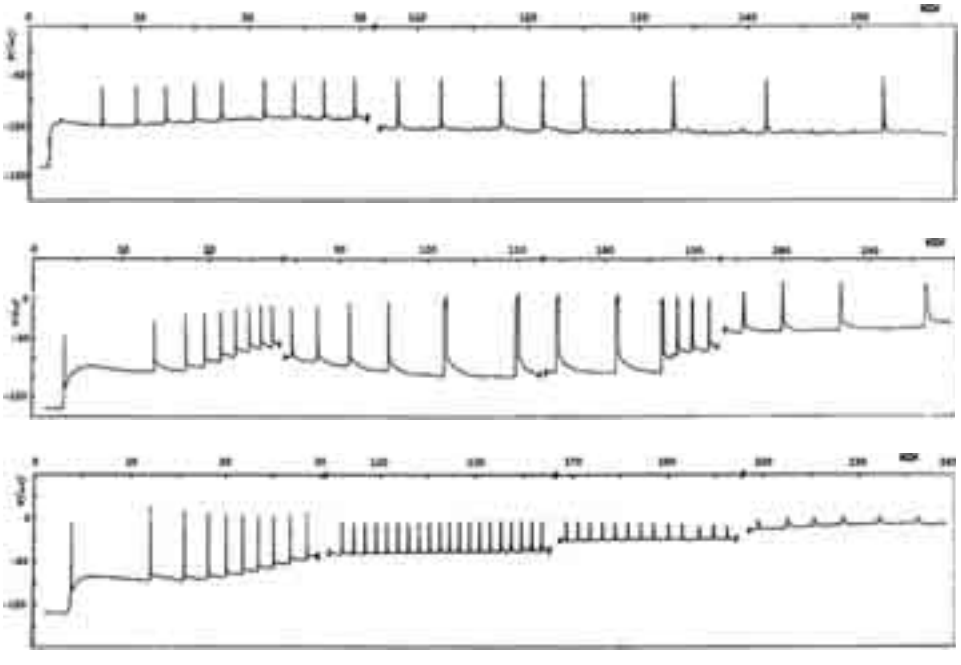

Fig. 4: Fourth class of oscillations. A-C: NH_4 ion concentration: 10 mM
For parameters see Tab. 5

ding of oscillations of the membrane potential was interrupted between the 30th and 105th minute and the analyses were performed separately. In both segments of oscillating, local impulses were observed among single impulses, provided that the number of local impulses was much greater in the second segment. The frequency of the occurrence of single impulses declined towards the end of oscillating, Figure 4 A, Table 5 A.

Tab. 5: Fourth class of membrane potential oscillations

	Conc. (mM)	Duration of membrane potential oscillation (min)	Duration of preoscillatory period (min)	Duration of oscillatory period (min)	Frequency of singular oscillation appearance (osc/min)	Relative amplitude of oscillation (mV)	Segment of oscillogram
A	10	156.34	4.72	151.62	—	—	whole
		29.14			0.31	X_{av} = 44.20	I
		51.34			0.16	X_{av} = 59.25	II
B	10	295.63	0.00	295.63	—	—	whole
		24.30			0.37	X_{av} = 72.32	I
		28.86			0.21	X_{av} = 99.28	II
		19.18			0.31	X_{av} = 98.28	III
		25.63			0.16	X_{av} = 66.00	IV
C	10	235.88	0.00	235.88	—	—	whole
		26.53			0.38	X_{av} = 78.70	I
		23.33			0.86	X_{av} = 38.62	II
		18.59			0.70	X_{av} = 21.61	III
		20.82			0.29	X_{av} = 9.49	IV

Oscillating presented in Figure 4 B and Table 5 B (10 mM NH_4Cl) was the longest registered oscillating of the membrane potential. Over 100 single impulses were generated in five hours. The frequency of generating of single impulses varied; the process of depolarisation occurred in the beginning and at the end of this oscillating, while gradual repolarisation occurred in the middle of oscillating. This oscillating was characterised by the irregular occurrence of impulses, Figure 4 B, Table 5 B.

Oscillating presented in Figure 4 C and Table 5 C (10 mM NH_4Cl) had the greatest number of generated single impulses (over 140). This oscillating started similarly to oscillating presented in Figure 8. The difference occurred in the magnitude of amplitudes of single impulses. This oscillating will also be analysed by its segments. It seems that segments II, III and IV had stable states and approximately equal amplitudes of single impulses, which is not characteristic for the majority of oscillations induced by the NH_4 ion. Oscillating ended with the occurrence of single impulses of low amplitudes and small frequencies, Figure 4 C and Table 5 C.

DISCUSSION

1. *General and specific characteristics of oscillations of the membrane potential induced by different NH_4Cl concentrations*

The first prerequisite for the occurrence of oscillations of the membrane potential is the excitation of the living cell and thereby its membranes. The excitation in these experiments was performed by the addition of different NH_4Cl concentrations to the external solution.

According to the obtained results, the probability for inducing oscillations of the membrane potential in experiments with high NH_4Cl concentrations (8—10 mM) amounted to 62%. As expected it was much more difficult, but not impossible, to obtain oscillations at lower and low NH_4Cl concentrations. Oscillations of the membrane potential were most often induced after the first exchange of the standard solution (Table I) for the NH_4Cl solution. In such cases oscillating of the membrane potential was the most intensive by amplitude magnitudes, number of single and local impulses, as well as by the duration and frequency of the occurrence (Tables 2—5). Furthermore, this did not exclude the possibility to induce oscillations in repeated exchange of the stated solutions. But even if they occurred, the characteristics of such oscillations were weak and numerous with single and local impulses. The duration of oscillations of the membrane potential induced by 6—10 mM NH_4Cl was very long and lasted, on the average, up to 100 min with generation of over 50 single impulses (Figures 3 C — 4 A-C), (Tables 4 C — 5 A-C). Damping of these oscillations was minimal (1.038), and the frequency of generation of single impulses was also quite low (0.74 im min^{-1}). The occurrence of local impulses was significant and gradually increased with an inevitable prolongation of the impulse intervals. However, oscillating ended with a cessation of generation of local impulses.

The longest oscillating of the membrane potential was obtained in these experiments — the excitable but living plant cell *Nitelle* generated over 100 single impulses in the course of five hours (Figure 4 B).

Figure 4 C presents oscillating of the membrane potential with the greatest number of generated single impulses. Over 140 single impulses were generated in this oscillating for four hours.

A certain „pre-oscillatory period" occurred in some of the stated oscillations of the membrane potential from the moment of the exchange of the standard solution for the NH_4Cl solution up to the beginning of oscillating (Figures 1, 2 C, 3 B and 4 B). The stated pre-oscillatory period lasted for several minutes (Tables 2, 3 C, 4 B and 5 B).

Single impulse amplitudes did not change to a greater extent in oscillations of the membrane potential induced by different NH_4Cl concentrations. However, its kinetics varied in the processes of depolarisation and repolarisation. Single impulses most often lasted differently, sometimes for 2 to 4 sec, and sometimes 6 to 8 sec.

2. *Functional dependence of the membrane potential and transport process across the cell membrane*

It is well known that the membrane potential depends on a complex ion transport across the cell membrane. This dependence is encompassed by Fick's law of diffusion, Using's criterion, Teorel, Nernst-Plank-Goldman equation (R a d e n o v i ć, 1974, 1998, 2001). Moreover, it is also known that two action forces: the strength of the concentration gradient and the strength of the electric gradient are considered in the case of transport processes across the cell membrane.

The ion transport across the excitable cell membrane is characterised by passive and active transport processes. The diffusion most often occurs as a dominant carrier of passive transport processes. It exhibits as simple, restricted and relieved. A simple diffusion consists of ion transport processes through the lipid bilayer of the membrane, or through pores in proteins and through pores in the lipid bilayer. A restricted diffusion occurs in the form of spatial ion process through pores with charged groups on proteins. At last, a relieved diffusion occurs in the form of ion transport processes with a movable carrier, fixed carrier and as the diffusion of the exchange. It is obvious that there are the following two initiators of the stated ion transport processes with dominant passive characteristics: the ion concentration gradient and the membrane potential gradient. Naturally, in the case of coupling of these two gradients, ion transport processes with characteristics of active ion processes across the excitable cell membrane can occur. Certainly, active transport processes occur in opposition to the chemical and electrochemical gradients (R a d e n o v i ć, 1998, 2001, 2003) as they require energy. These processes do not proceed independently, but always with the processes of ATP hydrolysis, i.e. on the account of energy accumulated in the macroenergetic constituents and their bonds with ATP, that is by ATPase.

Different characters of movements of proteins, lipids, pigments and other structures bound by the complex also contribute to the mechanism of total transport processes across the excitable cell membrane (R a d e n o v i ć, 1998, 2001, 2003). These movement characters in the excitable cell membrane can be: lateral movement (typical for proteins and lipids), rotational movement (typical for proteins specialized in ion transport processes) and so-called flip-flop movement (typical for lipids and proteins that regulate transport processes from one side of the excitable cell membrane to the other). When the degree of excitement of the cell membrane is higher, the stated characters of the movement (of lipids, proteins) are more significant in their intensity, dynamics and diversity, and the total transport processes are affected (K o y s et al., 1993, R a d e n o v i ć, 1998, 2001, 2003).

Considering the above stated on the dependence between the membrane potential and transport processes, it is often said that the membrane potential (by its intensity and kinetics) is a measure of the total transport processes occurring across the cell membrane. Hence, when the membrane potential reaches the stationary state, then ion transport processes across the membrane are uniform by the direction, intensity and charge. Furthermore, when the mem-

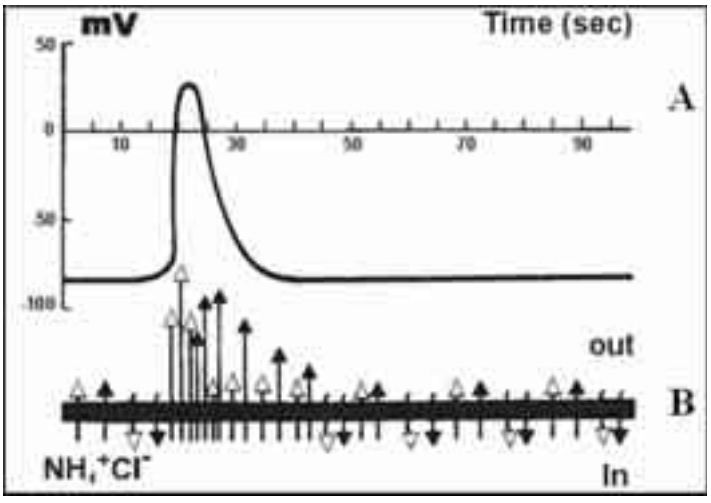

Fig. 5: Change of membrane potential during depolarization and repolarization (A) Schematic drawing of NH_4Cl ion oscillatory transport across the excitable cell membrane (B)

brane potential changes (linearly, exponentially, or in some other pattern), the ion transport processes occur in such dependence (by the direction, intensity and sign of the ion charge). Similar can be stated for the processes of depolarisation and repolarisation (Figure 5). It seems that a Cl^- transport and a partial NH_4 transport (presented by the direction, course, intensity and charge) occurred in the case of depolarisation processes (Figure 5). In cases of repolaristion, the NH_4 transport was dominant and Cl^- transport was partial (also presented by the direction, course, intensity and charge) (Figure 5). When the membrane potential was balanced, then Cl^- and NH_4 transports were also equilibrated (by the direction, course, intensity and charge) (Figure 5). Analogously to the stated, Cl^- and NH_4 transports occurred alternately in each single impulse of the oscillation of the membrane potential.

3. *Oscillatory pattern of transport processes across the excitable cell membrane*

The obtained results reported in this paper point out to the fact that oscillations of the membrane potential occur under certain conditions (Figures 1—4, Tables 2—5). Furthermore, a possible dependence of the membrane potential on different forms of ion transport processes across the excitable cell membrane is indicated. Therefore, based on the results and discussion, as well as, on our gained knowledge, we propose the following hypotheses:

— Oscillations of the membrane potential occur when the plant cell and thereby the cell membrane is excitable. As a rule, the excitable cell membrane is accompanied by unstable activities of ions: K^+, Na^+ and Cl^- that are no more constant in cell phases: vacuole, cytoplasm and cell wall (V o r o b l j e v et al, 1968, R a d e n o v i ć, 1983, 1985a, 1986, 1998, 2001).

— When the excitation of the cell membrane is produced by NH_4Cl concentrations, proteins oscillate in the cell membrane and rhythmically perform ion (Cl^- and NH_4) transport processes across the excitable cell membrane in the oscillatory regimes of the membrane potential (Figure 1—4). In such a state, ion (Cl^- and NH_4) transport processes become cooperative by which conformational-functional changes of active ion channels that spread and contract are induced (in the oscillatory regime) and in such a way rhythmically change total transport processes.

— The occurrence of oscillations of the membrane potential and ion (Cl^- and NH_4) transport processes across the excitable cell membrane inevitably produce oscillations in cell supply in energies: chemical, osmotic and electric. Likewise, the dependence of transport processes and the metabolism becomes oscillatory. This especially relates to oscillatory processes of autoregulation within the plant cell.

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МЕХАНИЗАМ ОСЦИЛАТОРНОГ ТРАНСПОРТА NH_4 ЈОНА КРОЗ ПОБУЂЕНУ ЋЕЛИЈСКУ МЕМБРАНУ

Чедомир Н. Раденовић[1, 2], Милош. В. Бељански[3], Георгиј. В. Максимов[4], Александар А. Калаузи[5], Милан Д. Дражић[6]
[1] Институт за кукуруз, Земун Поље, Биофизичка лабораторија, Србија и Црна Гора
[2] Факултет за физичку хемију, Универзитет у Београду, Београд, Србија и Црна Гора
[3] Институт за општу и физичку хемију, Београд, Србија и Црна Гора
[4] Московски државни универзитет М. В. Ломоносов, 11899 Москва, Русија
[5] Центар за мултидисциплинарне студије, Универзитет у Београду, Београд, Србија и Црна Гора
[6] Математички факултет, Универзитет у Београду, Београд, Србија и Црна Гора

Резиме

У овом раду дају се резултати типичних класа осцилација мембранског потенцијала, које су изазване побуђивањем ћелијске мембране слатководне алге *Nitella*, са различитим концентрацијама раствора NH_4Cl. Утврђено је постојање четири класе осцилација мембранског потенцијала, више различитих појединачних и локалних импулса, који се јављају у ритму. Познато је да су процеси осциловања мембранског потенцијала у директној зависности од осцилаторних транспортних процеса NH_4 и Cl јона кроз побуђену ћелијску мембрану. Осцилаторни процеси кроз живу ћелијску мембрану, изазвани NH_4 јоном, имају специфичне параметре: дужина трајања осцилације, број импулса у осцилацији, интензитет амплитуде, фреквенција, фактор пригушења и други, у поређењу са осцилаторним процесима изазваним другим моновалентним катјонима. Предложена је хипотеза могућег механизма осцилаторног начина транспорта NH_4 и Cl јона кроз побуђену ћелијску мембрану.

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Vesna R. Milankov, Jelena S. Stamenković, Ante A. Vujić*

Department of Biology and Ecology, University of Novi Sad
Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia and Montenegro
* e-mail: vesnam@ib.ns.ac.yu

GENETIC EQUILIBRIUM IN *CHEILOSIA VERNALIS* POPULATIONS (DIPTERA: SYRPHIDAE)

ABSTRACT: Genetic equilibrium of polymorphic enzyme loci from four natural populations of *Cheilosia vernalis* (Fallén, 1817) was tested. The study populations were from different regions of the Balkan Peninsula: Mediterranean area (Morinj, Montenegro: CVMOR), low mountain in the Pannonian plain (Fruška Gora, Serbia: CVFG), and two high Dinaric mountains (Durmitor, Montenegro: CVDUR; and Kopaonik, Serbia: CVKOP). Out of twelve enzyme loci analyzed for genetic variability, only four to six were polymorphic in the studied populations. For those loci, the observed and expected values of genotype frequencies were compared with chi-square goodness-of-fit tests. Analysis of deviations of phenotypic classes from Hardy-Weinberg equilibrium revealed significant differences at polymorphic loci of all populations except in CVKOP. This implied possible important influence of evolutionary mechanisms such as low migration rates, population substructuring and natural selection in creation and maintenance of genetic variability.

KEY WORDS: allozyme, *Cheilosia vernalis*, Hardy-Weinberg equilibrium, Syrphidae

INTRODUCTION

For a species, evolution involves changes in the genetic makeup of a population from generation to generation. Thus, genetic variability of populations is a measure of the evolutionary potential of species. Assessing independence of alleles within loci, Hardy-Weinberg equilibrium (HWE), has been an objective of evolutionary biology. Nonindependence is a starting point for determining what forces are responsible for variation and changes in allele frequencies (Shoemaker et al., 1998). The HWE departures may be caused by any of the factors that promote differential reproduction. Some factors related to population genetics are: small population size that makes a population susceptible to random genetic drift, non-random mating in subdivided or fragmented populations, differential selection, mutations, and/or biological factors, inclu-

ding differences in allele frequencies between sexes, age classes or year classes (Richardson et al., 1986; Pasteur et al., 1988).

One of the most intriguing hoverfly taxa is the genus *Cheilosia*, which is among the most diverse genera of the Palaearctic Syrphidae (Vujić, 1996), with over 400 Palaearctic species (Vujić, 1992), 175 of which are European (Speight, 2003). *Cheilosia* larvae are phytophagous or fungivorous, and it has been hypothesized that adaptive radiation at the larval stage gave rise to numerous cryptic and polytypic species (Vujić, 1996). Determination of *Cheilosia* species is difficult and often inconsistent (Speight, 2003) and the taxonomic status of many *Cheilosia* species remains uncertain. One such species is *Cheilosia vernalis*, a member of the *melanura* group (Vujić, 1996), which has been the subject of scientific debate ever since its description. Great variation of the morphological traits and distinct seasonal dimorphism (Vujić, 1992) spurred the taxonomic controversy, which resulted in 7 synonyms for *C. vernalis* (Peck, 1988).

Genetic studies of diploid organisms using allozyme electrophoresis are often based on co-dominant alleles at autosomal loci. Analysis of hierarchical organization and spatial and temporal variation of taxa by electrophoresis is a reliable and commonly used technique for quantifying the genetic variability of populations. Thus far, the study of genetic variation in *C. vernalis* populations from the Balkan Peninsula was based on the nuclear allozyme gene (Milankov et al. 2002a, 2002b) and mitochondrial DNA (mtDNA) sequence data (Ståhls et al., unpublished). These data revealed a large spatial variation of genotype and allelic frequencies at allozyme loci (Milankov et al., 2002a), strong influence of low migration rates and population substructuring (Milankov et al., unpublished), and likely presence of cryptic taxa (Milankov et al., unpublished; Ståhls et al., unpublished). Yet, potential factors responsible for the genetic divergence of the conspecific populations have not been determined.

Understanding the molecular basis of adaptation and quantified genetic variation in populations of *C. vernalis* elicited further research. The goal of this paper was to analyze the gametic equilibrium in populations of *C. vernalis* from the Balkan Peninsula, and try to elucidate potential factors driving the change in genotype frequencies of the studied populations.

MATERIAL AND METHODS

Sample collection

Samples of the species *Cheilosia vernalis* were collected from four regions of the Balkan Peninsula (population code and number of collected specimens in parenthesis): Mediterranean area (Morinj, Montenegro — CVMOR: 46 specimens), hilly area of the Pannonian plain (Fruška Gora, Serbia — CVFG: 26), and two high Dinaric mountains (Durmitor, Montenegro — CVDUR: 34, and Kopaonik, Serbia — CVKOP: 6). These regions span a variety of biomes, from intermixed evergreen Mediterranean maritime woodlands, and maquis

(Morinj); isolated deciduous woodlands on the slopes of a low mountain (Fruška Gora); to deciduous woodlands at low altitudes (up to 700 m), coniferous boreal woodlands at higher altitudes and alpine and high rocky pastures and snow patches in the highest zone on the mountain peaks of the two Dinaric mountains (Kopaonik and Durmitor). A more detailed description of the collection sites is given in Milankov et al. (2002a).

Allozyme analysis

Twelve isozyme loci (for details see Milankov et al., 2002a) were analyzed using 5% polyacrylamide gel electrophoresis according to Munstermann (1979) with slight modifications (Milankov, 2001).

Extracts from different body regions were used for electrophoresis depending on metabolic function and regional distribution of an enzyme (head in 0.10 ml of loading buffer: FUM, HK, IDH, MDH, PGM; thorax in 0.15 ml loading buffer: GPD, GPI, HK, IDH, SOD). Insect specimen electrophoresis was performed in the same gel for direct interpopulation comparison. Loci were numbered and alleles marked alphabetically with respect to increasing anodal migration.

Analysis

Genotype frequencies were obtained by direct genetic interpretation of bands on gels. Genetic interpretation was done using Mendel's rule of inheritance of codominant genes. Genetic variation was analyzed using the computer program BIOSYS-2 (Swofford and Selander, 1981, modified by Black, 1997).

Chi-square goodness-of-fit test was performed using the observed and genotype frequencies expected under the Hardy-Weinberg equilibrium. Expected genotype frequencies were adjusted by Levene's coefficient for small samples (Levene, 1949). When more than two alleles were observed at a locus, genotypes were pooled into three classes (all alleles except the most common one were treated as a single allele) and tested again. Three genotypes classes were calculated: (1) homozygotes for the most common allele, (2) heterozygotes for the most common allele and one of the other alleles and (3) all other genotypes.

RESULTS

Twenty eight alleles were identified at 12 analyzed loci (Milankov et al., 2002a). However, only four (CVMOR) to six loci (CVFG) were polymorphic in the studied populations (CVDUR and CVKOP had 5) (Milankov et al., 2002a). The results of the chi-square tests for deviations of observed genotypes from expected are summarized in Table 1.

Table 1. χ^2 statistic (degrees of freedom in parentheses) for deviation from Hardy-Weinberg equilibrium at polymorphic loci in four natural populations of *Cheilosia vernalis*. Levels of significance: *** = significant at $P \leq 0.001$, ** = significant at $P \leq 0.01$, or NS = $P \geq 0.05$

Locus#	CVMOR	CVDUR	CVKOP	CVFG
Gpi	76.73 (3) ***	29.67 (3) ***	1.97 (3) NS	35.31 (6) ***
Had	0.00 (1) NS	10.73 (6) NS	0.00 (1) NS	7.31 (3) NS
Hk-2, Hk-3	25.11 (1) ***	—	—	—
Idh-2	—	0.00 (1) NS	—	0.00 (1) NS
Mdh-1	—	31.01 (1) ***	2.33 (1) NS	—
Mdh-2	—	—	4.02 (1) **	20.51 (1) ***
Pgm	—	43.51 (6) ***	4.70 (3) NS	34.73 (3) ***
Sod-1	—	—	—	0.00 (1) NS

— denotes that a locus was monomorphic in the studied population;
#Gpi (glucose phosphate isomerase; E.C. 5.3.1.9), *Had* (2-hydroxy acid dehydrogenase; E.C. 1.1.99.6); *Hk-2, Hk-3* (hexokinase; E.C. 2.7.1.1), *Idh*-2 (isocitrate dehydrogenase; E.C. 1.1.1.42), *Mdh-1, Mdh-2* (malate dehydrogenase; E.C. 1.1.1.37), *Pgm* (phosphoglucomutase; E.C. 2.7.5.1), *Sod-1* (superoxide dismutase; E.C. 1.15.1.1)

Analysis of deviation of pooled phenotype classes from the equilibrium, for populations with degrees of freedom larger than one, revealed that the loci *Pgm* and *Had* were in disequilibrium in CVKOP and CVFG, respectively (Tab. 2).

Table 2. χ^2 statistic (degrees of freedom in parenthesis) for deviation from Hardy-Weinberg equilibrium of pooled phenotype classes in the populations of *Cheilosia vernalis*. Levels of significance: *** = significant at $P \leq 0.001$, ** = significant at $P \leq 0.01$, or NS = $P \geq 0.05$

Locus#	CVMOR	CVDUR	CVKOP	CVFG
Gpi	43.57 (1) ***	19.79 (1) ***	0.86 (1) NS	19.18 (1) ***
Had	—	0.55 (1) NS	—	8.15 (1) **
Pgm	—	26.43 (1) ***	3.22 (1) **	26.05 (1) ***

#Gpi (glucose phosphate isomerase; E.C. 5.3.1.9), *Had* (2-hydroxy acid dehydrogenase; E.C. 1.1.99.6); *Pgm* (phosphoglucomutase; E.C. 2.7.5.1)

DISCUSSION

At the *Gpi* locus, the studied populations of *C. vernalis* had the heterozygous combination characteristic for other syrphid populations, with „slow" and „fast" allelomorph (M i l a n k o v, 2001). Apart from a few populations in genus *Merodon*, in all hoverflies populations surveyed so far the genotype proportions for the *Gpi* locus showed statistically significant departures from the expectations of the Hardy-Weinberg equilibrium (M i l a n k o v, 2001; L u d o š k i, 2002). Possible cause for the deviation of genotype frequencies from the expected values at the *Gpi* locus might be the presence of a lethal recessive allele, differential survival due to selection pressure against the „slow" homozygotes, or inability to detect the activity of the allozyme coded by alleles in the homozygous combination. Significant deviation of the observed genotype frequencies from the expected at *Mdh-2* locus in the CVKOP population might be due to the presence of a cryptic taxon with specific combination of genotypes $Gpi^{a/a}$ and $Mdh\text{-}2^{c/c}$ (M i l a n k o v et al., 2002a). In population-genetic analysis it is very important to consider effects of random drift, especially in the case of small populations which are more likely impacted by changeable environment factors, like populations of *C. vernalis*. Temporal variation between samples collected in different years was observed in the CVMOR population. Unstable genetic structure registered in the small, isolated CVMOR population could be an indicator of possible bottleneck events (severe reduction of population size), or selection „against" certain alleles (= phenotypes) during a certain period of activity. Additionally, during the short period of adult activity (only a few days) in 1995, 1996 and 1997 in CVFG, only very small numbers of active adults were registered, probably due to the high mortality, caused by sudden changes in temperature. We could assume that the weather conditions may have caused a significant decrease of the effective population size (M i l a n k o v et al., 2002a).

Excess homozygosity, indicated by the high values of the Wright's Fixation index (F_{is}; W r i g h t, 1951) and Selander's *D* coefficient (S e l a n d e r, 1970) was observed in all populations at all loci, except at *Had* in CVMOR and CVKOP, *Idh-2* in CVDUR and CVFG and *Sod-1* in CVFG (M i l a n k o v et al., 2002a). It could be hypothesized that heterozygote deficiency at the majority of analyzed loci was caused by non-random mating within spatially fragmented populations, small effective population size (including effect of random process), disruptive selection, the impact factor in origins, and maintenance of genetic polymorphism of species, common for species that use the environment in roughly granulated form.

In a previous study of population-genetic structure of four populations of *C. vernalis*, genetic differentiation was quantified using Wright's F_{st} coefficient (W r i g h t, 1951) and Nei's genetic distance (N e i, 1978). A correlation between standardized allelic frequencies (F_{st}) and both geographic and genetic distance were observed. Furthermore, the analysis of genetic differentiation based on the allelic frequencies revealed that the occurrence of genetic changes during independent evolution of conspecific populations was not equal at all loci. This implies that the influence of gene flow, historical effects and genetic

drift were probably not important in genetic divergence. Contrary to this, different selective pressures on the individuals of the analyzed populations were probably a dominant mechanism of the genetic differentiation (Milankov et al., unpublished). Moreover, geographic distribution of the genotypes at *Gpi, Had, Idh-2, Mdh-2, Pgm* and *Sod-1* loci were recorded. Spatial variation was observed by registered *major*, *rare*, unique alleles and heterozygote genotypes (Milankov et al., 2002a).

In order to understand the adaptive relevance of the observed genetic polymorphism in populations of *C. vernalis* linkage disequilibrium was analyzed. The analysis of the allelic association of nine polymorphic loci in the spatially fragmented population of *C. vernalis*, the high percent of linkage alleles, ranging from 60% to 75%, was recorded. These results suggested that low migration rates, population substructuring and natural selection highly influenced the genetic divergence among the conspecific populations (Milankov et al., unpublished).

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ГЕНЕТИЧКА РАВНОТЕЖА У ПОПУЛАЦИЈАМА ВРСТЕ *CHEILOSIA VERNALIS* (*DIPTERA*: *SYRPHIDAE*)

Весна Р. Миланков*, Јелена С. Стаменковић, Анте А. Вујић
Департман за биологију и екологију, Универзитет у Новом Саду
Трг Доситеја Обрадовића 2, 21000 Нови Сад, Србија и Црна Гора
* e-mail: vesnam@ib.ns.ac.yu

Резиме

У раду је анализирана генетичка равнотежа полиморфних ензимских локуса четири популације врсте *Cheilosia vernalis* (F a l l é n, 1817). Анализиране популације воде порекло из четири различита региона Балканског полуострва: медитеранске области (Мориљ, Црна Гора: ЦВМОР), ниске планине Панонске низије (Фрушка гора, Србија: ЦВФГ), и две високе планине Динарида (Дурмитор, Црна Гора: ЦВДУР; и Копаоник, Србија: ЦВКОП). Од 12 анализираних ензимских локуса четири до шест је било полиморфно. У полиморфним локусима су упоређене израчунате и очекиване вредности генотипских фреквенција χ^2 тестом. Анализом одступања фенотипских класа од очекиваних вредности према Харди-Вајнберговом принципу утврђено је значајно одступање у свим полиморфним локусима свих популација осим у ЦВКОП популацији. Резултати упућују на вероватан утицај еволуционих механизама као што су смањени проток гена, популациона структурираност и природна селекција у креирању и одржавању генетичке варијабилности.

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Goran S. Marković, Predrag S. Veljović

Faculty of Agronomy Čačak, University of Kragujevac
Cara Dušana 34, 32000 Čačak, Serbia and Montenegro

BIOTIC INDICES TO BE USED FOR ASSESSMENT OF ICHTHYOFAUNA STRUCTURE OF THE ZAPADNA MORAVA RIVER (WEST SERBIA, THE DANUBE BASIN)

ABSTRACT: The presence of 25 fish species from 8 families was found to exist in the ichthyofauna of the Zapadna Morava river (the Danube basin, West Serbia) in the period from 1996—1999. The structure of the fish community was analysed using a substantial number of indices (Shannon's index α — diversity, Margalef's index of diversity, Sorensen's index of biotic similarity and Saprobic index of community). Qualitative-quantitative relationships in the ichthyofauna of the 4 river and one lake profiles were compared, too. High differences due to diversity in the general environmental conditions existing in the individual habitats were also established. An excessively high antropogenous impact on the diversity of aquatic ecosystems gave rise not only to a deteriorated water quality, but also to an abundance of the allochthonous ichthyofauna representatives.

KEY WORDS: Zapadna Morava, ichthyofauna, analysis, information indices

INTRODUCTION

Biological monitoring of the surface water courses has had a tradition for more than one century. At first, the experiences gained from utilisation of the aquatic organisms in bioindication were only haphazard to become more systematic later. The formation of saprobic systems, i.e. their classification depending on the degree of their presence in the waters being unevenly organic polluted, has completed biological monitoring to the highest possible level so far. Saprobic systems were elaborated in large numbers after the World War II (Pantle and Buck, 1955; Zelinka et al., 1959; Liebman, 1962), but the fullest one was attained by Sladecek (1973). When assessing the aquatic environment quality, this as well as the other saprobic systems worked out by Ortenndorfer and Hofrat (1983) have had the saprobic indices which include all the categories of hydrobionts along with fish. However, saprobic indices for the ichthyofauna have not been developed fully because the saprobic valences for all the freshwater fish have not been determined yet, so that neither the species indicative of the higher degree of organic pollution

(for polysaprobity) exist, nor the saprobically evaluated ones originating from the areas other than this, i.e. the allochthonous species (M a r k o v i ć, 1996).

In addition to saprobic indices, the ichthyofauna structure of a particular watercourse may be analysed using a higher number of information indices (R i c k e r 1975), thereby enabling the comparison of the qualitative-quantitative relationships between the populations in the individual sectors of the same ecosystem, as well as the considerations to be made on the alteration trend of this outstandingly dynamic component of aquatic biocenoses.

MATERIAL AND METHODS

Hydrobiological researches of the Zapadna Morava river were carried out in the period from 1996—1999. The 298 km long Z. Morava river represents one of the bigger tributaries of the Danube on the territory of Serbia. Ichthyological material was collected from 4 river profiles — Kratovska Stena (206 km of the river watercourse), Čačak (171 km), Kraljevo (104 km) and Jasika (20.5 km), as well as from the reservoirs Međuvršje (HE dam being at 182 km of the river course).

The fish were sampled by means of nets of different dimensions (from 1 x 1m to 50 x 2 m with mesh size 10 x 10 mm to 75 x 75 mm) with various fishing ancillaries being used. Identification of the samples caught was made by following the standard methods (V u k o v i ć and I v a n o v i ć, 1971; L a d i g e s and V o g t, 1979; W h e e l e r, 1983).

Shannon's index α — diversity (*H*) (K r e b s, 1994) was also determined:

$$H = -\sum\left(\frac{ni}{N}\right) \times Ln\left(\frac{ni}{N}\right)$$

where *ni* stands for the number of individuals of one species, *N* for the total number of individuals of all the species and *Ln* — for natural logarithm.

The diversity of the fish communities of single profiles was assessed using the Margalef's index of diversity (M a r g a l e f, 1958):

$$d = \frac{S-1}{LnN}$$

S — the total number of species, LnN — the natural logarithm of the total number of the individuals being caught.

Sorensen's index of biotic similarity (*S*) (S o r e n s e n, 1948) was used for comparing the composition of the fish habitats within the profiles being sought:

$$S = \frac{2c}{a+b}$$

where *a* stands for the number of fish species of the one profile, b stands for that of species of the other profile and c being the number of species common to both profiles.

Saprobic index of community (*S*) S l a d a c e k 1973 was determined on the basis of qualitative and quantitative composition and saprobic values of the individual species according to the formula, as follows:

$$S = \frac{\sum s \times h}{\sum h}$$

s — saprobic value of the species (S l a d a c e k, 1973),
h — relative abundance of the species (P a n t l e - B u c k, 1955).

RESULT AND DISCUSSION

The ichthyofauna of the Zapadna Morava comprised 25 fish species from 8 families in the period from 1996—1999 (M a r k o v i ć, 2002). The dominant family was *Cyprinidae* with 17 species (68%), *Percidae* with 2 species (8%), while those of *Siluridae, Esocidae, Cobitidae, Balitoridae, Centrarchidae* and *Ictaluridae* were monotypic. The family *Cyprinidae* dominated with its individual presence, so that of the entirely 4151 caught specimens, 3710 (90.67%) accounted for this family (Fig. 2). In terms of ichthyofaunitical components, the analysed sector of the Z. Morava river may be characterised as the watercourse of the C y p r i n i d a e character.

In addition to 21 autochthonous, 4 allochthonous (introduced) species, i.e. *Carassius auratus gibelio* and *Pseudorasbora parva* (originating from the Far East) and North American ones *Lepomis gibbosus* and *Ictalurus nebulosus* were also established in the fish community.

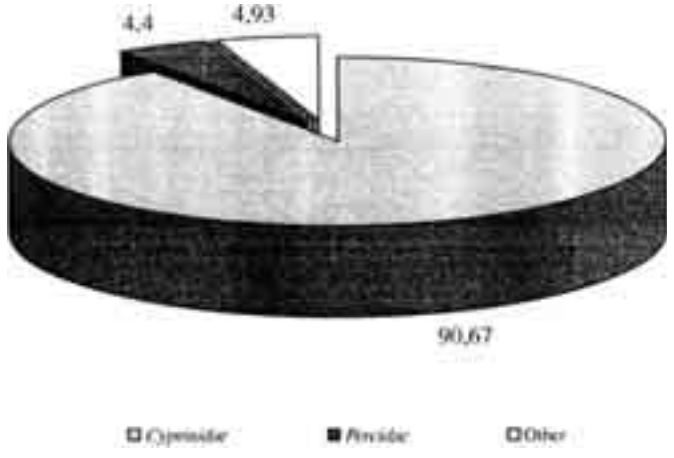

Fig. 1. The presence of the individual families in the Zapadna Morava ichthyofauna expressed in % (1996—1999)

Quantitatively, *Alburnus alburnus* accounting for 20.72% of the total catch (Tab. 1) was remarkably abundant. Thus, this species was revealed to have a high adaptibility to the worsened environemental conditions, dominating in all the profiles of the Z. Morava. A highly high abundance revealed *Chondrostoma nasus* to account for 10.79%, *Leuciscus cephalus* (10.48%) and *Rutilus rutilus* (9.47%) of a subdominant rank each. Also, *Carassius auratus gibelio* (6.63%) and *Abramis brama* (5.23%) were visibly abundant. The 6 species we have been concerned above account for 63.32% of the total sampled ichthyological material.

Tab. 1. The Zapadna Morava Ichthyofauna Composition in the Period from 1996—1999

Taxon	K. Stena		Čačak		Kraljevo		Jasika		Međuvršje		TOTAL	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
CYPRINIDAE												
Albumus albumus (Linnaeus, 1758)	85	23.88	103	13.15	117	18.40	75	14.42	480	25.86	860	20.72
Albumoides bipunctatus (Bloch, 1782)	22	8.33	87	11.11	33	5.19	25	4.81	—	—	177	4.26
Abramis brama (Linnaeus, 1758)	—	—	33	4.21	27	4.24	22	4.23	135	7.27	217	5.23
Abramissapa (Pallas, 1811)	—	—	—	—	—	—	19	3.65	10	0.54	29	0.70
Barbusbarbus (Linnaeus, 1758)	42	11.80	52	6.64	21	3.30	42	8.07	25	1.35	182	4.38
Barbus peloponnesius (Valenciennes, 1844)	12	3.37	41	5.24	47	7.39	29	5.58	—	—	129	3.11
Carassius auratus gibelio (Bloch, 1783)	—	—	20	2.55	11	1.73	32	6.15	212	11.42	275	6.63
Chondrostoma nasus (Linnaeus, 1758)	27	7.58	59	7.53	66	10.38	39	7.50	257	13.85	448	10.79
Cyprinus carpio (Linnaeus, 1758)	—	—	39	4.98	12	1.88	17	3.27	37	1.99	105	2.53
Aspius aspius (Linnaeus. 1758)	—	—	42	5.36	6	0.94	3	0.58	12	0.65	63	1.52
Gobio gobio (Linnaeus. 1758)	45	12.64	50	6.38	38	5.97	19	3.65	10	0.54	162	3.90
Leuciscus cephalus (Linnaeus, 1758)	42	11.80	99	12.64	71	11.27	69	13.27	154	8.30	435	10.48
Pseudorasbora parva (Schlegel, 1842)	—	—	7	0.89	15	2.36	32	6.15	120	6.46	174	4.19
Rhodeus sericeus amarus (Pallas, 1776)	—	—	10	1.28	—	—	—	—	50	2.70	60	1.44
Rutilus rutilus (Linnaeus, 1758)	31	8.71	52	6.44	93	14.62	42	8.07	175	9.43	393	9.47
Tinea tinea (Linnaeus, 1758)	—	—	2	0.25	2	0.31	2	0.38	5	0.27	11	0.27
Vimba vimba (Linnaeus, 1758)	—	—	27	3.44	11	1.73	7	1.34	—	—	45	1.08

SILURIDAE												
Silurus glanis (Linnaeus, 1758)	—	—	6	0.77	4	0.63	7	1.34	12	0.65	29	0.70
ESOCIDAE												
Esox lucius (Linnaeus, 1758)	1	0.28	10	1.28	3	0.47	6	1.15	11	0.59	31	0.74
COBITIDAE												
Cobitis taenia (Linnaeus, 1758)	—	—	1	0.13	6	0.94	—	—	2	0.10	9	0.22
BALITORIDAE												
Barbatula barbatula (Linnaeus, 1758)	27	7.58	5	0.54	—	—	—	—	—	—	32	0.77
PERCIDAE												
Perca fluviatilis (Linnaeus, 1758)	12	3.37	25	3.19	31	4.87	22	4.23	92	4.95	182	4.38
Zingel zingel (Linnaeus, 1766)	—	—	1	0.13	—	—	—	—	—	—	1	0.02
CENTRARCHIDAE												
Lepomis gibbosus (Linnaeus, 1758)	—	—	9	1.15	22	3.46	11	2.16	30	1.62	72	1.73
ICTALURIDAE												
Ictalurus nebulosus (Le Suer, 1819)	—	—	3	0.38	—	—	—	—	27	1.45	30	0.72
TOTAL	356	8.58	783	18.86	636	15.32	520	12.53	1856	44.71	4151	100.00

Increasing presence of the allochthonous fish species accounitng for 13.27% of the total ichthyofauna is doubtless by being particularly pronounced in the Međuvršje reservoir. The high presence of these undesired members of the fish community is endangering the viability of the autochthonous species. Hyperproduction of these fish species was noticed, contrasting a decreased number of the commercially more valued species (*Cyprinus carpio, Silurus glanis* and *Esox lucius*) altogether accounting for 3.97% the ichthyofauna of the sector studied. The disturbed relationships in the fish community structure are corroborated by a scarce presence of the obligatory predator species (*E. lucius, S. glanis* and *Aspius aspius*) — the total of 2.96% of the ichthyofauna being present.

As far as the fish community of the KRATOVSKA STENA profile is concerned, 11 species from 4 families were revealed, with highly dominating *A. alburnus* (23.88% of the total ichthyofauna presence), *Gobio gobio* accounting for 12.64%, *L. cephalus* and *Barbus barbus* accounting for 11.80%. The profile ichthyofauna diversity has been reduced by permanently polluted upstream watercourses (Đetinja, Moravica and Bjelica), as well as by the presence of HE plant Ovčar Banja thwarting the upstream migrations of the fish. Along with the dominance of *A. alburnus*, a relatively high abundance of the *Barbus* strain representatives and of those appearing abundantly in the watercourse of high and transitory type (*Alburnoides bipunctatis, G. gobio* and *Barbatula barbatula*) renders the profile ichthyofauna a barbel-cyprinidae charac-

ter. A low diversity of the species and individual presence led to the lowest values a — diversity (2.1666) and Margalef's diversity index (1.7022) in the ecosystem under way.

The profile ČAČAK comprises the most diversified fish community within the whole sector. Thus, 24 fish species were evidenced. By their number, *A. alburnus* (13.15%), *L. cephalus* (12.64%) and *A. bipunctatus* (11.11%) were found to be dominant. The high ichthyofauna diversity (the values α diversity of 2.7415 and diversity index of 3.4518) was favoured by habitat diversity enabling the survival of a higher number of the stagnophilous and reophilous species. Nevertheless, the ichthyofauna composition was not eligible due to domination of young individuals of the predators whose trophic range did not limit the hyper-production of trash fish. The best continuity attained with the ichthyological monitoring of this profile deserves mention.

The ichthyofauna of KRALJEVO was found to consist of 20 species, the most abundant being *A. alburnus* (18.40%), succeeded by *R. rutilus* (14.62%), *L. cephalus* (11.27%) and *Ch. nasus* (10.38%). A markedly more stable hydrological regimen, better water quality and a more developed macrozoobenthos community favoured the conditions of the ichthyofauna viability compared to the majority of other river profiles. The values of index α — diversity (2.5645) and of diversity index (2.9434) were lower compared to those in the previously depicted profile.

The profile JASIKA is inhabited by 20 fish species, with the dominance of *A. alburnus* (14.42%) and *L. cephalus* (13.27%). The local ichthyofauna composition was found to affected by the hydrological regimen and the Ibar river water quality. In addition, the profile ichthyofauna could merge with the fish assemblages of adjacently located watercourses of Rasina and Velika Morava. An approximated number of higher number of the species was on behalf of higher values α diversity (2.7197) and diversity indices (3.0382).

In the MEĐUVRŠJE reservoir, the presence of 20 fish species from 7 families was evidenced. The dominance of *A. alburnus* (25.86%) was profound, the subdominant species being *Ch. nasus* (13.85%) and *C. auratus gibelio* (11.42%). Eutrofication of the lake ecosystem favoured „trash" fish. A steady increase in the number of allochthonous species was observed, accounting for 20.45% of the profile ichthyofauna. On the other hand, obligate predators appeared in scarce number (only 3.23%) of the caught fish. The decline in number of this, commercially more valued species (*C. carpio* accounting for 1.99% of the ichthyofauna number), favours deterioration of the environmental conditions in the reservoir. This is also substantiated by an excessive catch during the spawn and other periods, despite fish care service. Originally reophilous species showing high adaptibility to this slowly flowing ecosystem, *Ch. nasus* and *L. cephalus*, were found in large numbers in the profile.

Diversity and spatial distribution of the sector ichthyofauna was highly variable. The lowest number of the species and that of the individuals sampled in the profile Kratovska Stena brought about the lowest diversity of the fish community (Shannon's and Margalef's index). The highest number of the species and the highest ichthyofauna diversity was observed in the profile Čačak. The remaining two river profiles (Kraljevo and Jasika) indicated likeness in

the number of registered fish species and similarity of the diversity indices values. The highest number of the individuals (the absence of some reophilous species) brought about the lower values of the indices found (Tab. 2).

Tab. 2. The fish habitat diversity of the analysed Z. Morava sector

Profile	No. species	Shannon's index α — diversity (*H*)	Margalef's index of diversity (*d*)
Kratovska Stena	11	2.1666	1.7022
Čačak	24	2.7415	3.4518
Kraljevo	20	2.5645	2.9434
Jasika	20	2.7197	3.0382
Međuvršje	20	2.3536	2.5245

Such trends also hold true for biocenotic similarity of the fish community (Tab. 3). The lowest biocenotic similarity was registered between the fish community of Kratovska Stena and that of Čačak (0.6286), as well as between the profiles of Kratovska Stena and the remaining ones (0.6452). A scarce diversity of the profile ichthyofauna resulted from the habitat general environmental conditions and local population detachment. Biocenotic similarity among the remaining river profiles was high (from 0.8182 to 0.9500) benefited by undisturbed migrations. A high biocenotic similarity characterised the ichthyofauna of both Međuvršje and other river profiles (0.8182—0.8500).

Tab. 3. Biocenotic similarity of the Zapadna Morava river ichthyofauna (Sorensen)

Profile	K. Stena	Čačak	Kraljevo	Jasika	Međuvršje
K. Stena		0.6286	0.6452	0.6452	0.6452
Čačak	0.6286		0.9090	0.8636	0.8182
Kraljevo	0.6452	0.9090		0.9500	0.8500
Jasika	0.6452	0.8636	0.9500		0.8500
Međuvršje	0.6452	0.8182	0.8500	0.8500	

The qualitative-quantitative analysis made of the fish habitat composition of the Z. Morava sector under way and the knowledge of saprobic values of the individual species enabled calculation of the ichthyofauna saprobic indices (Fig. 2). Saprobic indices of the river profiles were found to be in a narrow range from 1.95 to 1.99. Somewhat higher values were recorded in the profile of the Međuvršje reservoir (2.06) as a consequence of an abundance of stagnophilous species characterised by a higher saprobic value. The recorded values of saprobic indices were within the bounds of betamesosaprobity, but lower than those recorded for the plankton community.

Among the factors causing an unfavourable ichthyofauna structure of the analysed Z. Morava sector, deteriorated water quality, regulated river bed, negligence in river bottom exploitation, non-periodical work of the Međuvršje HE plant and turbulent water flow release, followed by an uncontrollable catch of the fish species, an inexpert and often haphazard fish planting deserve men-

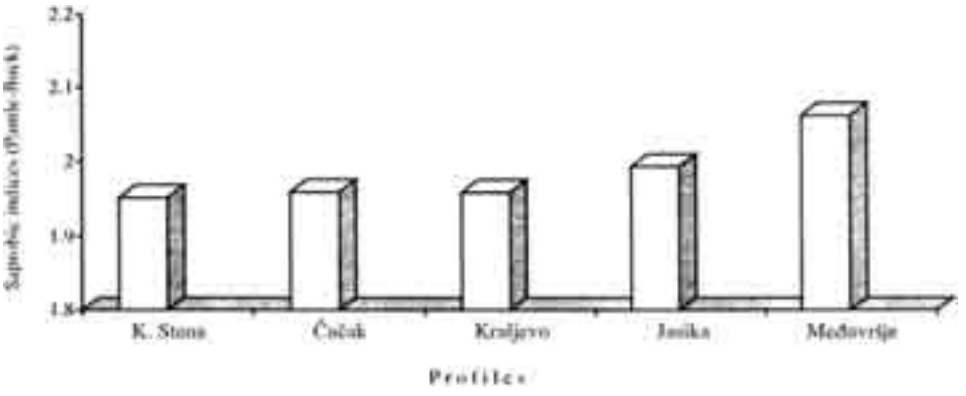

Fig. 2. Saprobic indices of the Zapadna Morava ichthyofauna (1996—1999)

tion. Some non-commercially valued species have proved a good adaptability to the worsened ecological conditions and are expanding at the cost of a decreased predator number. The high diversity of ichthyofauna of the single profiles is not only favoured by the abundantly increased species being adapted to organic pollution, but also by an increasing number of the allochthonous species whose viability is endangering the autchthonous ichthyofauna of the Z. Morava, thereby even more deteriorating the general hydrobiological conditions of the ecosystem.

CONCLUSIONS

Over the period from 1996—1999, the presence of 25 fish species from 8 families was established in the ichthyofauna of the Zapadna Morava river (the Danube basin, West Serbia). The fish community structure was analysed following a larger number of information indices. Qualitative-quantitative relationships pertaining to the four river and one lake profiles were also compared. The differences which had resulted from the diversity in the general environmental conditions of the individual habitats were visible. Lastly, a huge antropogenous impact on this component of the aquatic ecosystem was revealed in terms of considerably deteriorated water quality and highly abundant allochthonous ichthyofauna species.

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ПРИМЕНА БИОТИЧКИХ ИНДЕКСА У ОЦЕНИ СТРУКТУРЕ ИХТИОФАУНЕ РЕКЕ ЗАПАДНЕ МОРАВЕ (ЗАПАДНА СРБИЈА, ДУНАВСКИ СЛИВ)

Горан С. Марковић, Предраг С. Вељовић
Агрономски факултет Чачак
Универзитет у Крагујевцу
Цара Душана 34, 32000 Чачак, Србија и Црна Гора

Резиме

У периоду 1996—1999. године констатовано је присуство 25 врста из 8 фамилија у ихтиофауни реке Западне Мораве (Дунавски слив, Западна Србија). Стуктура рибље заједнице је анализирана применом већег броја информационих индекса (Шеноновог индекса α — диверзитета, Маргалефовог индекса разноврсности, Соренсеновог индекса биотичке сличности и Сапробног индекса заједнице). Упоређивани су квалитативно-квантитативни односи у ихтиофауни 4 речна и једног језерског профила. Установљене разлике су условљене диверзитетом општих еколошких услова појединих станишта. Изразито велик антропогени утицај на ову компоненту акватичних екосистема, поред погоршања квалитета воде, посебно је испољен у омасовљењу алохтоних представника ихтиофауне.

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Dušan D. Stojadinović,[1] *Zoran N. Nikić,*[2] *Duško M. Isaković*[3]

[1] Institute for the Development of Water Resources „Jaroslav Cerni", P. O. Box 33—34, Belgrade, Serbia and Montenegro
[2] Faculty of Forestry, University of Belgrade, Kneza Višeslava 1, Belgrade, Serbia and Montenegro
[3] Agency for Maintenance and Development of Inland Waterways, Francuska 9, Belgrade, Serbia and Montenegro

HYDRO-GEOLOGICAL PROPERTIES OF THE SAVIAN AQUIFER IN THE COUNTY OBRENOVAC

ABSTRACT: The paper presents a description of hydrogeological researches of alluvial layers of the Sava River in the area of the source „Vic Bare" near Obrenovac. This source supplies groundwater to that town. The depth of these layers amounts to 25 m. With regard to collecting capacity, the most significant are gravel-sand sediments of high filtration properties. Their average depth amounts to about 13 m with the underlying layer made of Pleistocene clays. Compact aquifer is formed within these sediments and it refills partly from the Sava River at places where river cuts its channel into the gravel-sand layer. The analysis of the groundwater regime in the riparian area points out that groundwater levels follow stages of the Sava River. Such an influence lessens with the distance. Established hydraulic connection between the river and the aquifer enables its permanent replenishment. On the other hand, due to certain pollutions this river flow might bring along, it represents a potential danger. Those pollutions could enter water-bearing layer of the aquifer as well as the exploitation well of the source. Such presumptions have been confirmed in the experiment of pollution transport carried out in the water-bearing layer. Unabsorbable chloride was used as a tracer whose movement velocity through exploitation well proved that there were real possibilities of intrusion of aggressive pollutants into the water-bearing layer and into the aquifer as well. Therefore, the protection of the source must be in the function of the protection of surface waters.

KEY WORDS: aquifer, pollution, protection, river

INTRODUCTION

On the territory of Republic of Serbia, the Sava River makes an extensive alluvial plain 3—15 km wide and more than 80 km long. Such an extensive natural amphitheatre enabled the formation of an abundant aquifer, known in literature as the Savian aquifer. According to hydro-geological researches, alluvial formations of this aquifer consist of gravel-sand and sand sediments that

represent basic collector of the groundwater, while fine-grained and slurry sands and clays make its roof layer. The depth of the water-bearing layer varies from 12 m to 20 m, sporadically 25 m. This depth lessens with the distance toward the rim. Gravel-sand sediments, as a collector, have good filtration properties with the infiltration rate of 10^{-4} m/s. Water-impermeable clays make an underlying stratum to these highly water-impermeable formations. Replenishment of the collecting water-bearing layer is carried out by precipitation over the wide plain and inflow from the groundwater that comes from the rim part, especially from the north-east and south rim. To some extent, existing irrigation canal network can also provide water to the aquifer.

However, main inflows of the water to this aquifer come from the surface water of the Sava. The intensity of that inflow depends on the degree of the cutting of the Sava's channel into gravel-sand sediments. The analysis of the groundwater regime in riparian area points out that water table follows water levels of the Sava, while this influence weakens with the distance. Generally, statical, piezometric groundwater level is 3 m bellow the ground, while in the zone of exploitation wells dynamic level is 3.5—4.0 m below the surface of the ground. Significant depth of alluvial formations, favorable filtration characteristics of the water-bearing layer and the way of its replenishment enabled the occurrence of an abundant groundwater reservoir that is thoroughly in use. There are 31 tubular and two Ranny wells in the zone of the source „Vic Bare". Their total discharge is 400 l/s. Mutual radius of influence of these wells is about 150—200 m. After drawing, groundwater is subjected to chlorination, demanganisation and defferisation in order to be brought into the state of usability.

MATERIAL AND METHODS

The source „Vic Bare" is the best example for the comprehension of hydrogeological properties of alluvial formations in this area. It is located between the Sava River and the village Zabrežje north of Obrenovac (Figure 1).

With regard to litho-stratigraphy, two significantly characteristic mediums are distinguished (Figure 2):

— Pleistocene sediments (clay)

— Holocene sediments (gravel-sand sediments laying directly over Pleistocene clays)

Pleistocene clays, as water impermeable layers, are significant hydro geologic isolators with the transmission coefficient of 10^{-7} m/s or less. Holocene sediments, which have a recipient function, can be divided into two zones: lower, roughly porous zone consisted of arenaceous gravels and sands of average depth 13 m and less porous zone of average depth 4.5 m that consists of slurry sands and silty and clayey sands that represent roof layer to hydrogeological collector. Sediments from the lower zone distinguish with inter-granular porosity, with the compact type of the aquifer with free flow that is formed within. Transmission coefficient of the water-bearing layer, calculated in accordance with granulometric composition, is 2.5—8.5 x 10^{-4} m/s, while the value of transmission coefficient, calculated through the experiment, is 5—7 x

Figure 1. Geographic position of the source Vic Bare

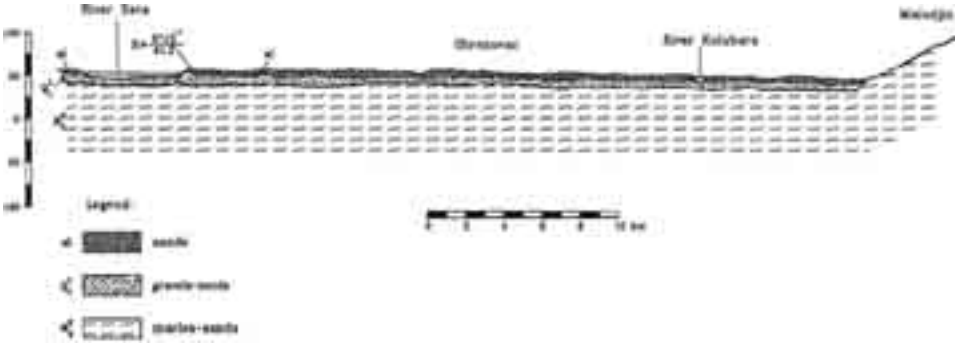

Figure 2. Geologic-lythological profile Mislođin-Obrenovac

10^{-4} m/s. The average value of the transmission coefficient for the whole Holocene waterbearing complex, calculated on the basis of grain size distribution curve, is 4.5 x 10^{-4} m/s. The source „Vic Bare" belongs to the wide Savian aquifer that replenishes by precipitation over relatively thin roof layer and by water from the Sava river at the places where the river has cut its channel into the gravel-sand layer, whereby there has been established good hydraulic connection between surface and underground flow.

With regard to physical properties of these waters, they distinguish with increased opacity; they are without odour, taste, with pH value of 7.8. Electrical conductivity of these waters is 505.3 μS/cm, while hardness is 17.0° dH. Their chemical composition is shown in Table 1.

Table 1. Chemical composition of groundwater from source „Vic Bare"

N°	Element	Content (mg/l)
1	Calcium (Ca^{++})	86.88
2	Magnesium (Mg^{++})	60.97
3	Sodium (Na^{+})	36.42
4	Potassium (K^{+})	1.07
5	Chlorides (Cl^{-})	12.00
6	Sulfates (SO_4^{-})	29.44
7	Nitrates (NO_3^{-})	0.11
8	Nitrites (NO_2^{-})	0.00
9	Ammonia (NH_4)	1.15
10	Iron (Fe)	1.50
11	Manganese (Mn)	0.22
12	$KMnO_4$	7.90
13	Dry residue	414.00

RESULTS AND DISCUSSIONS

Considering possible accidental pollutions in the Sava River, maintenance of the quality of the groundwater from this aquifer is a permanent task. Seeing that the water quality in this river can be worsened by excessive disposal of waste and industrial waters, it can directly affect the quality of the groundwater. These intrusions are particularly significant at the places with established good hydraulic connection between surface and underground flow. Carried out experiment on the transport of the pollution within the water-bearing layer of this aquifer confirmed such a statement (Figure 3). This test was conducted in situ by direct insertion of the tracer into the water-bearing layer. Unsorbable chloride was used as a tracer. It was funneled into tentative piezometer P-1 and its transport was controlled at the control piezometer P-2. The test was carried out under conditions of the exploitation well B-2. The basic task of this experiment was to determine the dispersion coefficient and sorbic parameters. On the basis of gained values, the dispersion coefficient (D) amounted to

6.88 x 10^{-4} m/s, while the velocity of the tracer-pollutant (Vcl) through the water-bearing layer was 1.76 x 10^{-3} m/s. Considering the collecting layer of good filtration properties, where the course of groundwater movement is toward exploitation wells, there is a possibility of transportation of certain pollutants by the groundwater flow into the fields of exploitation wells.

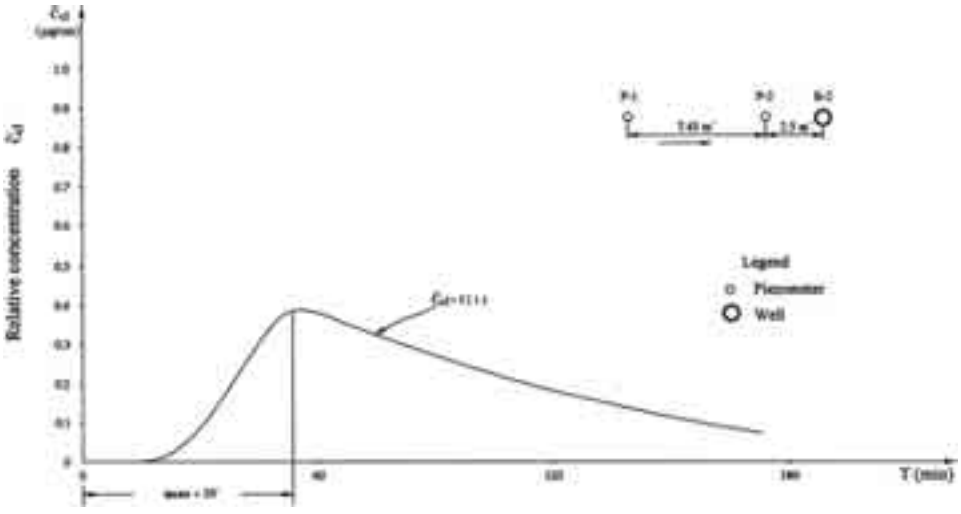

Figure 3. Results of the experiment on funneling tracer into the groundwater of the well B-2

CONCLUSION

Alluvial formations of the Savian aquifer and their properties point out that they are subject to possible contamination by untreated waste and industrial waters and other spilled pollutants if they are disposed of into the flow or into the alluvial plain of the Sava River. Therefore, protection of groundwater must be paid particular attention and care, especially through the zones of sanitary protection.

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ХИДРОГЕОЛОШКЕ КАРАКТЕРИСТИКЕ ПОСАВСКОГ АКВИФЕРА НА ДЕЛУ ОБРЕНОВЦА

Душан Д. Стојановић,[1] Зоран Н. Никић,[2] Душко М. Исаковић[3]
[1] Институт за водопривреду „Јарослав Черни”,
Р. О. Вох 33—34, Београд, Србија и Црна Гора
[2] Шумарски факултет, Универзитет у Београду,
Кнеза Вишеслава 1, Београд, Србија и Црна Гора
[3] Установа за одржавање и развој унутрашњих пловних путева,
Француска 9, Београд, Србија и Црна Гора

Резиме

У раду се даје приказ хидрогеолошких својстава алувијалних наслага реке Саве на подручју изворишта „Вић Баре” код Обреновца. Из овог изворишта град се снабдева подземном водом. Од алувијалних наслага, чија укупна дебљина износи и до 25 m, најзначајнију колекторску улогу имају песковито-шљунковити седименти добрих филтрационих карактеристика. Њихова просечна дебљина износи 13 m, са падином изграђеном од плеистоцених глина. У оквиру њих је формиран збијени тип издани, чије се прихрањивање, једним делом, врши и из речног тока Саве на местима где је река усекла своје корито у шљунковито-песковити слој. Анализа режима подземних вода у приобалном појасу реке Саве указује да нивои подземних вода прате водостаје Саве, да би са удаљавањем од ње тај утицај постепено слабио. Успостављена хидрауличка веза између реке и издани омогућује, са једне стране, њено перманентно прихрањивање, а са друге стране представља потенцијалну опасност да одређена загађења, која овај речни ток може да носи са собом, дођу у издански водоносни слој, а тиме и у поље експлоатационог бунара изворишта. Ове претпоставке су потврђене и изведеним експерименталним опитом транспорта загађења, извршеним у изданском водоносном слоју. Као трасер је коришћен несорбирајући хлорид, чија је брзина кретања кроз експлоатационо поље показала да постоје реалне могућности продора агресивног загађивача у водоносни слој, а тиме и у извориште. Услед тога заштита подземних вода мора бити у функцији и заштите површинских вода.

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Mirjana M. Kresović,
Svetlana B. Antić-Mladenović,
Vlado Đ. Ličina

University of Belgrade, Faculty of Agriculture,
Nemanjina 6, Zemun, Serbia and Montenegro

AEROBIC AND ANAEROBIC INCUBATION — BIOLOGICAL INDEXES OF SOIL NITROGEN AVAILABILITY

ABSTRACT: Our researches have been made on brown forest soil that had been used in long-term experiments set up according to specified fertilization system for over 30 years. We have chosen those experiment variants in which quantities of nitrogen fertilizers were gradually increased. The soil samples taken from 0 cm to 30 cm depth were used to determine biological indexes of nitrogen availability (aerobic and anaerobic incubation). The same samles were also used for pot experiments with oat. Plant and soil parameters obtained in controlled conditions were used for determination of biological indexes reliability in measuring the soil nitrogen availability. On the grounds of correlation analysis, it can be concluded that biological index of nitrogen availability achieved by the anaerobic incubation (without substraction of the initial content of available nitrogen) of the investigated brown forest soil is the reliable indicator of soil nitrogen availability. That is not the case with the aerobic incubation in which reliability has not been established.

KEY WORDS: aerobic incubation, anaerobic incubation, biological index, nitrogen, plant and soil parameters, availability

INTRODUCTION

Biological methods used to determine the soil nitrogen availability index have been researched and considered to be quite reliable in assessing the soil nitrogen availability by a number of authors (Keeney and Bremner, 1966; Ozus and Hanway, 1966; Robinson, 1968; Stevanović, 1978; Confort and Walmsley, 1971; Gasser and Kalembasa, 1976).

Aerobic incubation is a satisfactory method in assessment of the plant nitrogen availability, considering the fact that nitrogen mineralization during incubation is being caused by the same organisms that mineralize nitrogen in the field. Although this is a good argument, we cannot neglect the fact that the en-

vironment conditions (humidity, temperature, aeration) that are being monitored during mineralization in laboratory do significantly differ from the ones in the field. Despite the limitations that we mentioned, many researchers have established that the aerobic incubation procedure can give suitable results (Allison and Sterling, 1949; Fits et al., 1953; Munson and Stanford, 1955; Robinson, 1968 a, b; Stanford and Lagg, 1968; Fox and Piekielek, 1978; Power, 1980; Stajković, 1990).

In 1964, taking into consideration the mentioned limitations of the aerobic incubation, Waring and Bremner suggested a new method called anaerobic incubation to determine the nitrogen availability index.

This method, in comparison with aerobic method, has the following advantages:

Only NH_4-N content is being estimated, the initial quantity of NO_3-N is lost by denitrification, while further nitrification is completely stopped, bigger quantity of nitrogen is being developed in a shorter period of time (7 days) than in an aerobic incubation. That means that this is a faster method, which is important in routine analyses.

Anaerobic incubation, together with the aerobic one, has been the most commonly applied procedure among the biological methods used to determine the nitrogen availability index. The values obtained through these procedures (NH_4-N and NO_3-N in aerobic and NH_4-N in anaerobic) are most frequently compared to the plant parameters (yield, N intake or N% in the cultivated plants), whether the plants have been cultivated in control conditions (pots) or in the field.

The aim of our researches was to verify the value of aerobic and anaerobic methods, as the most significant biological methods in assessment of soil nitrogen availability. The researches have been done on brown forest soil that had been used in long-term experiments set up according to specified fertilization system for over 30 years.

MATERIAL AND METHODS

The researches have been done on brown forest soil (Mladenovac) that had been used in long-term experiments carried out by the Institute for soils in Belgrade. The long-term experiments are set up according to specified fertilization system with mineral fertilizers for over 30 years.

Experiment variants with increasing doses of nitrogen fertilizer were selected and applied in our investigations: control (Ø), $N_1P_2K_2$ (N_1-60, P_2-120, K_2-120 kg P_2O_5 and K_2O/ha), $N_2P_2K_2$ (N_2-90 kg/ha) $N_3P_2K_2$ (N_3-120 kg/ha) and $N_4P_2K_2$ (N_4-150 kg/ha). The samples from the mentioned experiment variants were taken in March for both aerobic and anaerobic incubation, as well as for the pot experiments.

I. Methods applied to estimate nitrogen availability

a) *Aerobic incubation*

250 ml glass jars, into which 10 g of air-dried soil was weighed, were used. The soil humidity was brought at 30%, the jars were covered with plastic foil. Incubation lasted for 14 days at the temperature of 30°C (B r e m n e r, 1965). After that, in an hour shaking, a 2 M KCl solution extraction was taken. Short lasting distillation in the presence of MgO and $CaCl_2$ and titration with 0.0025 H_2SO_4 were applied to determine the contents of NH_4-N and NO_3-N.

b) *Anaerobic incubation*

Five grams of air-dried soil were measured, put into tube and 12.5 ml of distilled water was added. Completely anaerobic conditions were achieved that way. The period of incubation lasted for 7 days at the temperature of 40°C (W a r i n g and B r e m n e r, 1964). After that, content from the tube was quantitatively transferred to distillation flask by multiple rinsing with 12.5 ml of 2 M KCl. Distillation was carried out with MgO and $CaCl_2$ and distillate libated was held in boric acid. Titration was done with 0.0025 M H_2SO_4 and the quantity of obtained NH_4-N was determined.

II. Methods used to establish chemical properties of the soil

Basic agochemical analysis of soil samples were taken from the long--term experiment were carried out using the following methods:

— Soil reaction: pH value in suspension with water and 1 M KCl was determined potentiometrically with glass electrode;

— Organic carbon and humus were determined using dichromatic method by Tjurin, modification of Simakov;

— Total nitrogen quantity was determined using semi-micro Kjeldahl method where the soil sample was digested with concentrated H_2SO_4 with the addition of catalyst mixture K_2SO_4: $CuSO_4$: Sn-1-1:10:100 (B r e m n e r, 1965)

— Content of available nitrogen was determined by steam distillation method from soil salt extract obtained after one-hour shaking with 2 M KCl. NH_4-N content was established through a short distillation in the presence of small quantities of MgO and $CaCl_2$. Distillate was caught in boric acid and titrated with 0.0025 M H_2SO_4. When NH_4-N had been determined, Devarda's alloy was added and new distillation was performed. This time NH_4-N was held in a new quantity of boric acid and titration with 0.0025 M H_2SO_4 was used to establish the contents of NO_3-N.

— Available phosphorus and potassium were established using Al-method by E g n e r - R i e h e m (1960).

III. Experiment in pots

Plastic pots, each containing 2 kg of soil from the layer 0 — 30 cm of soil, were used in the experiment. The soil was taken out in spring (March) from the labeled experiment variants in the field. Before the beginning of the experiment, soil was brought up to the air-dried conditions, ground and the desired quantity was measured. The vegetation experiment was performed with two fertilizing variants, the PK and NPK. Prior to sowing, the soil was mixed with the fertilizers that had previously been dissolved in water, i.e. with: NH_4NO_3, KH_2PO_4 and KCl. The used NH_4NO_3 was labeled with a stabile isotope ^{15}N (11.8%). In the PK variant 50 mg of P_2O_5 and K_2O/kg of soil was used, while in the NPK variant 50 mg of N, P_2O_5 and K_2O/kg of soil was used. Ten plants of oats per pot were used in the experiment. The plants were grown to the phase when plants blade. During the experiment, the humidity was maintained at level 60 to 80% of water retention capacity.

IV. Parameters of plants and soil in pots

Having in mind the most commonly used parameters for plants in pots, the following parameters were used in our researches to assess the values of the applied methods (Keeney and Bremner, 1966; Sirota, 1973; Peterson et al. 1960 and Sapošnjikov's suggestions, 1973): plant parameters (yield of oats in NPK and in PK experiment variants, relative increase in yield (PK = 100), relative increase in yield in NPK variant (Ø = 100), relative increase in yield in PK variant (Ø = 100), difference in yield (NPK-PK), difference in yield (NPK-Ø), content of nitrogen in cultivated oats (in NPK and PK)) and soil parameters (total uptake of nitrogen (NPK); uptake of soil nitrogen (NPK), uptake of fertilizer nitrogen (NPK), ratio of soil and fertilizer nitrogen uptake and uptake of soil nitrogen (PK)).

The simple linear correlation analysis was used to establish all the mentioned parameters of the plants and soil in pots, as well as for the biological methods applied (aerobic and anaerobic).

Absolute values of the nitrogen developed after both aerobic and anaerobic incubation procedures, with and without taking into account the initial content of available nitrogen (NH_4 and NH_3-N in aerobic and NH_4-N in anaerobic incubation) were used as biological indexes of nitrogen availability.

RESULTS AND DISCUSSION

Brown forest soil (Mladenovac) was used in these researches. Basic chemical properties of the investigated soil are presented in Table 1.

Table 1 — Chemical properties of the investigated soil

Variants	pH		Total nitrogen (%)	Humus %	C:N	Available		
	H2O	1 MKCl				P_2O_5	K_2O	NH_4+NO_3 (ppm)
						(mg/100 gr)		
Control	5.40	4.60	0.10	1.43	8.5	6.4	16.2	4.9
$N_1P_2K_2$	5.10	4.30	0.10	1.67	8.7	18.0	21.8	8.4
$N_1P_2K_2$	5.00	4.20	0.11	1.81	9.3	14.4	21.8	15.4
$N_3P_2K_2$	4.90	4.15	0.10	1.85	9.5	16.0	25.0	12.2
$N_4P_2K_2$	5.00	4.05	0.12	1.88	9.4	12.5	21.8	51.3

On the grounds of the obtained soil pH values (M KCl) the conclusion can be drawn that the investigated brown forest soil belongs to the acid or very acid soil category.

According to the humus content, the brown forest soil comes to the category of soils poor in humus, while its total nitrogen content puts it at the limits of poor content. The C/N ratio is somewhat lower (less than 10) than in (standard) arable soils due to the fact that drop in humus exceeded the drop of the total nitrogen content.

The content of available phosphorous varied in the field experiment variants. The lowest was in the control variant (6.4 mg/100 gr) and the highest was in the variant $N_1P_2K_2$ (18 mg/100 gr), which had put the soil into the poor to medium soil category.

Quantities of available nitrogen varied in the experiment variants in the regular pattern starting with the control experiment variant (4.9 ppm) to $N_4P_2K_2$ variant (51.3 ppm).

The aerobic incubation procedure used in these researches was introduced by Bremner (1965) as modification of some previous procedures (Allison and Starling, 1949; Fitts at al., 1953; Hanway and Dumenil, 1953).

It was Bremner's suggestion to calculate the quantity of the mineral nitrogen obtained during aerobic incubation by subtracting the established quantities of available nitrogen after and before incubation.

However, quite frequently quantities of the established mineralized nitrogen prove to be lower after incubation than before the incubation, particularly with the poorly fertile soils or with the soils with high content of residual mineral nitrogen. Hence, the quantity of mineralized and nitrified nitrogen may have negative values. One can suppose that such results are caused by immobilization. When soils of poor fertility are being used for the laboratory experiments, such conditions are favourable for mineralization processes. However, the obtained mineral nitrogen is being used by microorganisms so that the final count gives negative results. On the other hand, in the soils with higher contents of available nitrogen which, as a rule, originates from the previous fertilization (Herron at al., 1977), in conditions of optimum humidity and temperature, microorganisms will also develop and take up the available nitrogen in a more intensive way, so that the obtained results will have negative values in this case, too.

The conclusion may be drawn that negative results, when initial content of available nitrogen is subtracted, are the results of: disturbed balance between the process of mineralization and immobilization and too short incubation period (two weeks) for reestablishing balance between those two processes.

Due to all mentioned facts and suspicions connected to the origin of aerobic incubation (Stanford and Smith, 1972) on the one side and very precise Bremner's instructions (1965) regarding keeping and preparation of the soil samples on the other side, dilemma remains whether or not to subtract the initial content of the available nitrogen.

Because of the above-mentioned suspicions and dilemmas regarding calculation of the results, both procedures, i.e. nitrogen availability indexes were used in our investigations — initial condition was both subtracted and not subtracted.

Table 2 presents the quantities of mineralized and nitrified nitrogen in aerobic procedures, both with the initial content subtracted and not subtracted.

The quantity of mineralized and nitrified nitrogen in aerobic procedure obtained without subtraction of the initial content of available nitrogen points to the conclusion that there are no significant regularities in either increase or decrease mineralized and nitrified nitrogen in experiment variants.

Table 2 — Quantities of mineralized and nitrified nitrogen in aerobic procedure, initial content of the available nitrogen in the brown forest soil both subtracted and not subtracted

Experiment variants	Initial content of available nitrogen not subtracted	Initial content of available nitrogen subtracted		
	Quantities of mineralized and nitrified NH_4+NO_3-N (ppm)	Before NH_4+NO_3-N (ppm) incubation	After NH_4+NO_3-N (ppm) incubation	Quantities of mineralized and nitrified NH_4+NO_3-N (ppm)
Control	24.7	4.9	24.7	19.8
$N_1P_2K_2$	24.2	8.4	24.2	15.8
$N_1P_2K_2$	32.2	15.4	32.2	16.8
$N_3P_2K_2$	30.6	12.2	30.6	18.4
$N_4P_2K_2$	27.8	51.3	27.8	–23.5

Similar situation was also obtained with the quantities of mineralized and nitrified nitrogen when we subtracted the initial quantity. The only difference was that $N_4P_2K_2$ experiment variant gave negative value (–23.5 ppm), i.e. the quantity of mineralized nitrogen was smaller after incubation than before it. Therefore, one may suppose that during the incubation a part of available nitrogen gets immobilized by microorganisms.

An experiment in controlled conditions was carried out with the aim to assess the value and reliability of nitrogen availability biological indexes (obtained by aerobic and anaerobic incubation) through the plants' and soil's parameters.

Table 3 presents the results of the plants' and soil's parameters obtained in the controlled conditions.

Table 3 — Plants' and soil's parameters in the controlled conditions (in pots).

Plants and soil parameters / Experiment variants	Control	$N_1P_2K_2$	$N_2P_2K_2$	$N_3P_2K_2$	$N_4P_2K_2$
Yield (NPK) (g/pot)	10.26	10.84	10.86	11.22	11.10
Yield (PK) (g/pot)	2.25	3.58	4.18	4.69	6.36
Relative increase in yield (PK = 100)	456	303	260	239	174
Relative increase in yield (NPK) (Ø = 100)	100	106	106	109	108
Relative increase in yield (PK) (Ø = 100)	100	159	186	208	283
Difference in yield (g/pot) (NPK-PK)	8.01	7.26	6.68	6.53	4.74
Difference in yield (g/pot) (NPK-Ø)	—	0.58	0.60	0.96	0.84
Difference in yield (g/pot) (PK-Ø)	—	1.33	1.93	2.44	4.11
Total uptake of nitrogen (NPK)	90.0	105.8	118.0	110.1	115.1
Uptake of soil nitrogen (NPK)	58.1	72.4	83.8	78.0	84.4
Uptake of fertilizer nitrogen (NPK)	31.9	33.4	34.2	32.1	30.7
Ratio of soil and fertilizer nitrogen uptake	1.8	2.2	2.4	2.4	2.7
Uptake of nitrogen (PK)	18.5	22.9	30.3	30.1	48.5

The majority of authors have used absolute values, i.e. quantities of mineralized and nitrified nitrogen when applying aerobic incubation, i.e. biological indexes of nitrogen availability.

In our researches we have also compared absolute values with plants' and soil's parameters. In order to assess the value, i.e. reliability of aerobic incubation in estimation of the soil nitrogen availability, we have calculated the correlation coefficients.

Table 4 presents the values of correlation coefficients between mineralized and nitrified nitrogen in aerobic procedure.

Table 4 — Correlation coefficient between the plants' and soil's parameters and mineralized and nitrified nitrogen in aerobic procedure, the initial content of available nitrogen in the brown forest soil both subtracted and not subtracted

Plants and soil parameters	Quantities of mineralized and nitrified N, initial content of N subtracted Biological ind. I	Quantities of mineralized and nitrified N, initial content of N not subtracted Biological ind. II
Yield (NPK)	NS	NS
Yield (PK)	NS	NS
Relative increase in yield (PK = 100)	NS	–0.54*
Relative increase in yield (NPK) (Ø = 100)	NS	NS
Relative increase in yield (PK) (Ø = 100)	0.81**	NS

Difference in yield (g/pot) (NPK-PK)	NS	NS
Difference in yield (g/pot) (NPK-Ø)	NS	NS
Difference in yield (g/pot) (PK-Ø)	0.81**	NS
N(%) in plants (NPK)	NS	0.56*
N(%) in plants (PK)	NS	NS
Total uptake of nitrogen (NPK)	NS	0.54*
Uptake of soil nitrogen (NPK)	NS	0.57
Uptake of fertilizer nitrogen (NPK)	–0.62*	NS
Ratio of soil and fertilizer nitrogen uptake	NS	NS
Uptake of nitrogen (PK)	NS	NS

** significant at probability level 0.01
* significant at probability level 0.05
NS not statistically significant

Statistically significant correlation dependence between plants' and soil's parameters and aerobic incubation of available nitrogen was established in just a few cases and it was closer to low than to medium correlation dependence of minor statistical significance (r-(–0.54*), 0.56*, 0.54 and 0.57).

Statistically significant correlation between an aerobic incubation with initial content of available nitrogen subtracted and the plants' and soil parameters was also established in just a few cases. A high correlation dependence was established only in the relative increase of yield in the PK experiment variant (Ø = 100) and regarding the difference in yield (P-Ø). The value of coefficient was r = 0.81**. Medium negative correlation dependence was established in the uptake of fertilizer nitrogen (r = –0.62*).

As we have already mentioned, anaerobic method, together with aerobic one is the most commonly applied procedure used to establish biological indexes of the soil nitrogen availability.

In the research process, the quantity of the obtained NH_4-N in both anaerobic and aerobic procedures was calculated without subtracting the initial content of NH_4-N. However, the calculation procedure suggested by Waring and Bremner (1964) was also used, i.e. the initial NH_4-N content before the incubation was subtracted from the NH_4-N content established after the incubation.

Table 5 presents quantities of mineralized nitrogen that were established in anaerobic procedure, with the initial content both subtracted and not subtracted.

The quantities of mineralized nitrogen obtained without subtraction of the initial content of available nitrogen can be used as grounds for the following conclusion: there is a regular increase in the quantity of mineralized nitrogen, starting from the control variant and going towards the variant with the highest dosage of fertilizer nitrogen.

Table 5 — Quantities of nitrogen mineralized in anaerobic procedure, the initial content of NH_4-N available in brown forest soil subtracted and not subtracted

Experiment variants	Initial content of available NH_4-N not subtracted	Initial content of available NH_4-N subtracted		
	Quantities of mineralized nitrogen (NH_4-N, ppm)	Before NH_4-N incubation (ppm)	After NH_4-N incubation (ppm)	Quantities of mineralized nitrogen NH_4-N (ppm)
Control	14.8	3.8	14.8	11.0
$N_1P_2K_2$	18.0	6.6	18.0	11.4
$N_2P_2K_2$	17.7	12.6	17.7	5.1
$N_3P_2K_2$	19.7	7.5	19.7	12.2
$N_4P_2K_2$	20.1	45.5	20.1	–25.4

Quantities of nitrogen mineralized in anaerobic procedure, with the initial content of available NH_4-N counted out, considerably varied with the field experiment variants. No regularity was observed regarding the increase or decrease of mineralized nitrogen quantities going from the control variants towards the highest dosage of the applied fertilizer nitrogen. Negative values for the mineralized nitrogen quantities were established as in the aerobic incubation in the $N_4P_2K_2$ experiment variant.

The established absolute values regarding mineralized nitrogen, with the initial content of available NH_4-N both subtracted and not subtracted, were compared to the plants' and soil's parameters. The obtained correlation dependences were used to assess how reliable this method is in estimation of soil nitrogen availability.

Table 6 presents the values of correlation coefficients between the plants' and soil's parameters and nitrogen mineralized in anaerobic procedure, with the initial content of NH_4-N in the brown forest soil both subtracted and not subtracted.

Table 6 — Correlation coefficients between the plants' and soil's parameters and mineralized and nitrified nitrogen in anaerobic procedure, the initial content of available nitrogen in the brown forest soil both subtracted and not subtracted

Plants' and soil parameters	Quantities of mineralized nitrogen, initial NH_4-N content subtracted	Quantities of mineralized nitrogen, initial NH_4-N quantity not subtracted, biological ind. II
Yield (NPK)	NS	0.70**
Yield (PK)	–0.79**	0.90**
Relative increase in yield (PK = 100)	0.61*	–0.92**
Relative increase in yield (NPK) (Ø = 100)	NS	NS
Relative increase in yield (PK) (Ø = 100)	NS	0.81**
Difference in yield (NPK-PK)	0.80**	–0.74**
Difference in yield (NPK-Ø)	NS	NS
Difference in yield (PK-Ø)	NS	0.80**

N(%) in plants (NPK)	NS	NS
N(%) in plants (PK)	NS	–0.54
Total uptake of nitrogen (NPK)	NS	0.67**
Uptake of soil nitrogen (NPK)	–0.53**	0.75**
Uptake of fertilizer nitrogen (NPK)	NS	NS
Ratio of soil and fertilizer nitrogen uptake	–0.76**	0.88**
Uptake of nitrogen (PK)	–0.91**	0.77**

** significant at probability level 0.01
* significant at probability level 0.05
NS not statistically significant

Statistically significant correlation dependence between the plants' parameters and nitrogen mineralized in anaerobic procedure, with the initial content of NH_4-N subtracted, was not found in major number of cases, except for the yield obtained in the PK variant (r = 0.79**) and regarding the difference in obtained yield (NPK-PK) (r = 0.80**), where it was high. The relative yield increase (PK-100), using the method mentioned, gave medium correlative dependence (r = 0.61*). However, the remaining plants-in-pots-parameters in anaerobic incubation did not give statistically significant correlation dependences. The uptake of soil nitrogen in the NPK experiment variant in pots, with the nitrogen mineralized in anaerobic procedure, gave the medium negative correlation dependence (r = –0.53*). Nevertheless, a considerable negative correlation dependence was established (r = –0.91**) between the uptake of nitrogen in the PK variant and the method mentioned.

The values of correlation coefficients established for the obtained yields in the PK and NPK variants corresponded to the results obtained by Gasser and Kalembasa (1976). It is also the case with the uptake of nitrogen in those experiment variants. However, our researches resulted in negative correlation dependences as the increased available contents of ammoniacal nitrogen in soil have directly influenced the process of mineralization in anaerobic conditions. There was more nitrogen in the soil, mineralization was less intensive, while the yield and uptake of nitrogen were higher, because the plants have mainly used the available ammoniacal nitrogen, already present in the soil, to satisfy their nitrogen needs.

As it can be seen from the results presented in Table 6, a high and very high correlation dependence of major statistical significance was established between the nitrogen mineralized in anaerobic procedure (the initial content of NH_4-N not subtracted) and the plants-in-pots-parameters. A medium negative correlation dependence (r = –0.54*) was only established between the content of nitrogen in oat plant (PK) and the nitrogen mineralized in just mentioned procedure. Mainly high correlation dependence of major statistical significance was established between the soil in pots and the mineralized nitrogen.

The percentage of correlation dependences for r = 0.50—0.90, with significance of 0.05—0.01 probability level and for r = 0.70, with significance of 0.01 probability level was calculated on the grounds of the established correlation coefficients values for both methods used, i.e. two ways used to calculate the results.

This calculation was done with the aim to define clearly which of the nitrogen availability biological indexes that were used can be considered reliable to estimate nitrogen availability. The aim was also to determine which is the most suitable procedure to calculate results in aerobic and anaerobic incubation.

Table 7 presents the correlation coefficients expressed in percentages (in aerobic and anaerobic incubations), for both criteria.

Table 7 — Correlation dependences (in percents) between the plants and soil parameters and nitrogen availability biological indexes when r = 0.50—0.99 (**,*) and when r = 0.70**.

Parameters	Percentage of correlation dependence for r = 0.50—0.99 (**, *)	Percentage of correlation dependence for r ≥ 0.70**
	Aerobic incubation, the initial content of available nitrogen not subtracted	
Plants and soil in pots parameters	26.7	0.0
	Aerobic incubation, the initial content of available nitrogen subtracted	
Plants and soil in pots parameters	20.0	13.3
	Aerobic incubation, the initial content of available NH_4-N not subtracted	
Plants and soil in pots parameters	73.3	60.0
	Aerobic incubation, the initial content of available NH_4-N subtracted	
Plants and soil in pots parameters	40.0	26.7

As presented in the Table 7, the highest percentages of correlative dependence, for both criteria, were established for anaerobic incubation when initial content of available NH_4-N was not subtracted. However, considerably lower percentages were established for the same method when initial condition was not subtracted.

As opposed to anaerobic incubation, low percentages of correlative dependence were established in aerobic incubation for both calculation procedures.

CONCLUSIONS

The results presented here may be used to conclude the following:

Anaerobic incubation, i.e. the established biological index of availability in the investigated brown forest soil can be considered reliable in assessing the soil nitrogen availability.

Considering that in anaerobic incubation significantly higher percentages of correlation dependences of available NH_4-N were determined when the initial content of available NH_4-N was not subtracted than in the cases in which the initial content of available NH_4-N was subtracted, nitrogen availability biological index determined in anaerobic procedure without subtraction of the initial content of available NH_4-N, can be recommended to be used.

Both plants' and soil's in parameters obtained by experiment in pots can be used on an equal level to assess the value of the investigated nitrogen availability biological index.

The established reliabilities of biological index are significant for estimation of the soil nitrogen availability because anaerobic procedure is simple and quick from an analytical view and it can be used in routine analyses.

Nitrogen availability biological index established in our researches after the aerobic incubation, with the initial content of available nitrogen both subtracted and not subtracted, cannot be considered reliable to estimate the soil nitrogen availability.

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АЕРОБНА И АНАЕРОБНА ИНКУБАЦИЈА — БИОЛОШКИ ИНДЕКСИ ПРИСТУПАЧНОСТИ АЗОТА ЗЕМЉИШТА

Мирјана М. Кресовић, Светлана Б. Антић-Младеновић, Владо Ђ. Личина
Универзитет у Београду, Пољопривредни факултет,
Немањина 6, Земун, Србија и Црна Гора

Резиме

Истраживања су обављена на гајњачи (Младеновац) која се користи у оквиру дугогодишњег стационарног огледа, са одређеним плодоредом и системом ђубрења већ више од тридесет година. За ова истраживања одабиране су варијанте огледа где је примењена растућа доза азота ђубрења. За утврђивање биолошких индекса приступачности азота примењене су аеробна и анаеробна метода са два различита начина обрачуна резултата (са одузимањем и без одузимања почетног садржаја приступачног азота). Ради одређивања параметара биљака и земљишта који су вредновали поузданост коришћених метода за оцену приступачности азота земљишта изведен је оглед у контролисаним условима уз примену изотопски обележеног азота (^{15}N). На основу урађене корелационе анализе односно утврђене корелативне зависности између биолошких индекса и параметара биљака и земљишта може се закључити да се биолошки индекс који је утврђен анаеробним поступком без одузимања почетног садржаја приступачног NH_4 може сматрати поузданим за оцену приступачности азота земљишта. Поузданост није утврђена за аеробну методу (са одузимањем и без одузимања почетног садржаја приступачног азота) као и за анаеробни поступак са одузимањем почетног садржаја приступачног NH_4-N.

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Ksenija J. Taški-Ajduković[1], *Dragana M. Vasić*[2]

[1] National Laboratory for Seed Testing, M. Gorkog 30, 21000 Novi Sad, Serbia and Montenegro
[2] Institute of Field and Vegetable Crops, M. Gorkog 30, 21000 Novi Sad, Serbia and Montenegro

DIFFERENT STERILIZATION METHODS FOR OVERCOMING INTERNAL BACTERIAL INFECTION IN SUNFLOWER SEEDS

ABSTRACT: During culture of protoplasts in agarose droplets, permanent problem was bacterial infection. It was assumed that the seeds are the origin of infection, so different sterilization methods were tested in order to overcome this problem. Germination, infection of seeds and hypocotyls and their growth were examined. Based on these parameters, the best result was obtained with the combined use of 5% commercial bleach and dry heating at 45°C.

KEY WORDS: bacterial infection, seeds, sterilization, sunflower, tissue culture

INTRODUCTION

A critical stage in the introduction of plants to tissue culture is to obtain cultures free of microbial contamination. In spite of the surface sterilization process carried out for explants before culture, microbial growth inside the plant cannot be eliminated (Hennerty at al., 1988), particularly when explants are excised from field grown plants (Savela and Uosukainen, 1994) and transferred to *in vitro* culture.

Contaminants in the xylem vessel, which are protected from surface sterilization are endophytic bacteria (Hallman et al., 1997). Endophytic bacteria have probably evolved a close relationship with their host plant through co--evolutionary processes and may influence plant physiology in ways that have not yet been elucidated (Misaghi and Donndelinger, 1990). Inside the plant they have very little microbial competition (Misaghi and Donndelinger, 1990) and usually do not cause visible symptoms to the plant (Hallman et al., 1997; Peñalver et al., 1994). The bacteria may stay latent or symptomless (Peñalver et al., 1994) up to several months after

the initiation of culture and may not survive outside the plant tissue (Reed et al., 1995). Endophytic bacteria may even promote beneficial effects for field grown crops, but in stress conditions such as *in vitro* culture, latent endophytic bacteria may become pathogenic and detrimental to the growth and development of the plantlets (Leifert et al., 1989).

During culture of protoplasts in agarose droplets, permanent problem was bacterial infection. It was assumed that seeds are the origin of infection, so different sterilization methods were tested in order to overcome this problem.

MATERIAL AND METHODS

Plant material

Seeds of inbred line PH-BC_2-91A and Ha-74A of cultivated sunflower were obtained from Institute of Field and Vegetable Crops.

Sterilization methods

Different sterilization methods were tested:

1. soaking seeds in 70% ethanol for one minute followed by soaking in 14% commercial bleach for 20 minutes; rinsed tree times in distilled water; removing the seed coats; soaking seeds in 70% ethanol for one minute followed by soaking in 14% commercial bleach for 15 minutes; rinsed tree times in sterile distilled water
2. soaking seeds in 14% commercial bleach for 20 minutes; rinsed tree times in distilled water; removing the seed coats; soaking seeds in 5% commercial bleach for 60 minute; rinsed tree times in sterile distilled water
3. soaking seeds in 14% commercial bleach for 20 minutes; rinsed tree times in distilled water; removing the seed coats; soaking seeds in 14% commercial bleach for 15 minutes; rinsed tree times in sterile distilled water; heat sterilization at 45°C during 60 minutes
4. soaking seeds in 14% commercial bleach for 20 minutes; rinsed tree times in distilled water; removing the seed coats; soaking seeds in 5% commercial bleach for 60 minutes; rinsed tree times in sterile distilled water; heat sterilization at 45°C during 60 minutes
5. soaking seeds in 14% commercial bleach for 20 minutes; rinsed tree times in distilled water; removing the seed coats; soaking seeds in 5% commercial bleach for 60 minutes; rinsed tree times in sterile distilled water; heat sterilization in water bath at 45°C during 60 minutes.

The experiments were set in 6 repetitions with 6 seeds. The seeds were germinated for 2 days in the dark at 25°C. Germination of seeds and infection were followed.

Germinated seeds without infection were placed on a MS medium (Murashige and Skoog, 1962) and cultured in the dark at 25°C. After 7 days of culture infection of hypocotyls and their growth were examined.

All results were expressed as mean ± standard error (SE). Statistical analysis was performed by the analysis of variance (ANOVA), and posthoc comparisons between means were made by Duncan's multiple range test. Statistical significance was defined as being at the level $p < 0.05$.

RESULTS AND DISCUSION

During culture of protoplasts in agarose droplets, a permanent problem was internal bacterial infection, different methods were tested in order to overcome this problem. Other authors also report problems with internal bacterial infection in plant tissue culture (Hennerty at al., 1988; Misaghi and Donndelinger, 1990).

Besides sterilization of seeds with chemicals, the surface sterilization can be performed by exposure of seeds to UV light or heat. Since UV irradiation can damage DNA, seeds were sterilized according to 5 different protocols with commercial bleach and dry and moist heating. *Percent age* of germinated seeds (Fig. 1) and *percent age* of seed infection (Fig. 2) were followed, as well as growth (Fig. 3) and infection of hypocotyls (Fig. 4).

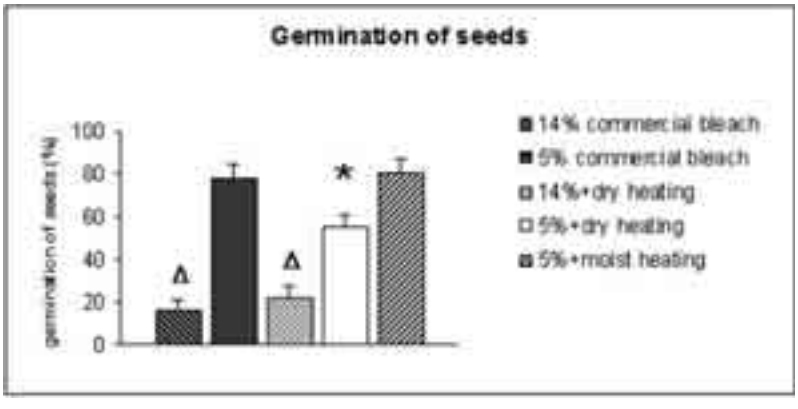

Figure 1. Germination of the seeds. Significance: *$p < 0.05$ vs. protocol 2, 4 and 5; $^{\Delta}p < 0.05$ vs. protocol 2 and 5

Based on the obtained data, germination of sunflower seeds was significantly lower after sterilization by 14% commercial bleach (Fig. 5). Significantly lower germination of seeds was also found after sterilization by combination of 5% commercial bleach and dry heating, when compared to the seeds sterilized by 5% commercial bleach and combination of 5% commercial bleach and dry heating (Fig. 1).

Seeds that were sterilized by dry heating (5% commercial bleach + dry heating and 14% commercial bleach and dry heating) were not infected. The infection of seeds was significantly reduced with these sterilization methods, when compared to the sterilization by 14% commercial bleach and 5% commercial bleach + moist heating (Fig 2.).

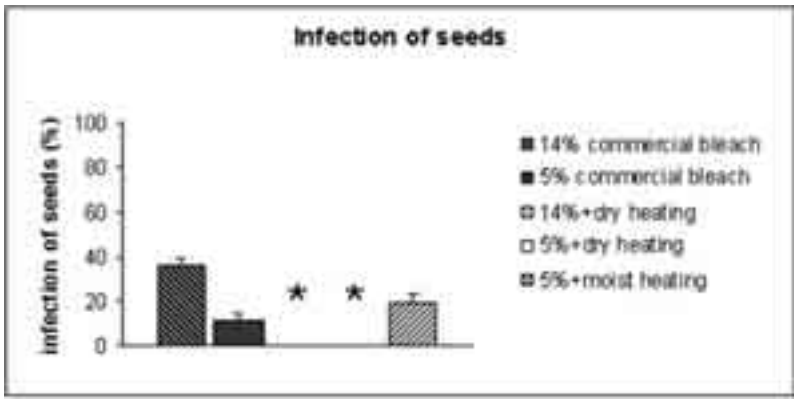

Figure 2. Infection of the seeds. Significance: *p < 0.05 vs. protocol 1 and 5

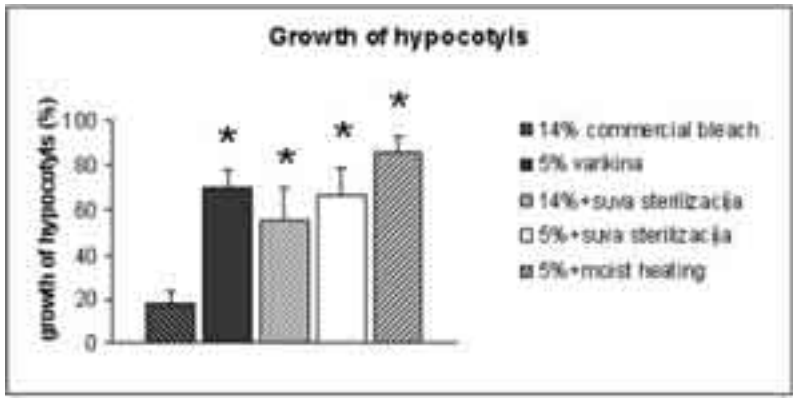

Figure 3. Growth of the hypocotyls. Significance: *p < 0.05 vs. protocol 1

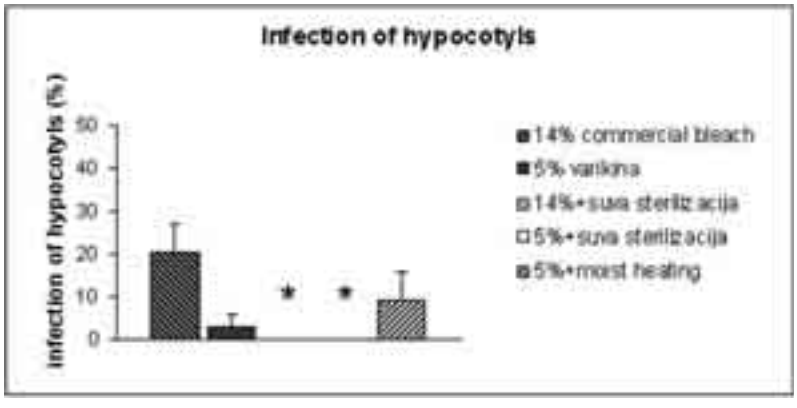

Figure 4. Infection of the hypocotyls. Significance: *p < 0.05 vs. protocol 1

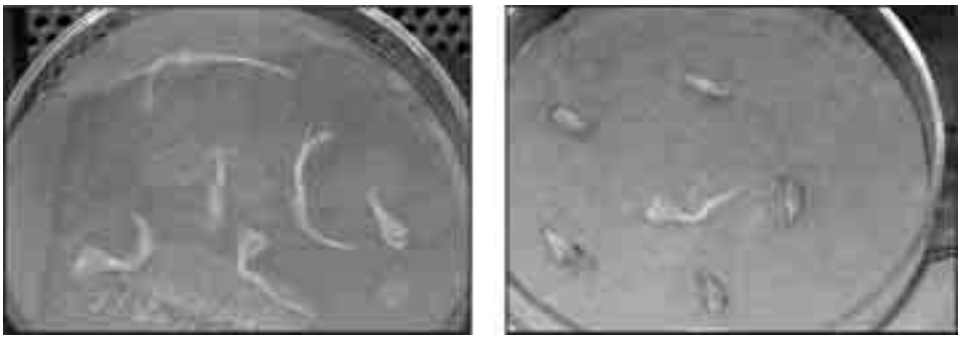

Figure 5. Germination of the seeds sterilized by 5% commercial bleach (left) and 14% commercial bleach (right)

Figure 6. Growth of the hypocotyls after seed sterilization by 14% commercial bleach (right) and combination of sterilization by 5% commercial bleach and dry heating (left)

Growth of hypocotyls after the sterilization of seeds by 14% commercial bleach was significantly lower, when compared to the other protocols (Fig 6.), and also when compared to the protocols with combination of sterilization by 14% commercial bleach + dry heating (Fig 3.).

After seed sterilization according to the protocols with dry heating (5% commercial bleach + dry heating and 14% commercial bleach and dry heating) hypocotyls were not infected (Fig 4.). However, with those methods infection of the hypocotyls was significantly reduced when compared only to the sterilization of seeds by 14% commercial bleach.

Similar results were obtained by inbred line Ha-74A.

The obtained results showed that combination of sterilization by 5% commercial bleach and dry heating gives the best results in overcoming problems

with internal bacterial infection. Thus it could represent a good method to obtain plants free of microbial contamination for tissue culture.

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УПОТРЕБА РАЗЛИЧИТИХ МЕТОДА СТЕРИЛИЗАЦИЈЕ СЕМЕНА СУНЦОКРЕТА У ПРЕВАЗИЛАЖЕЊУ ЕНДОГЕНЕ БАКТЕРИЈСКЕ ИНФЕКЦИЈЕ

Ташки-Ајдуковић Ј. Ксенија[1], Васић М. Драгана[2]
[1] Национална лабораторија за испитивање семена,
М. Горког 30, 21000 Нови Сад, Србија и Црна Гора
[2] Научни институт за ратарство и повртарство,
М. Горког 30, 21000 Нови Сад, Србија и Црна гора

Резиме

Приликом култивације протопласта гајеног сунцокрета у капљицама агарозе сталан проблем је била бактеријска инфекција. Како је претпостављено да је семе извор ове инфекције, испробане су различите методе његове стерилизације да би се покушао превазићи овај проблем. Праћени су клијавост семена, број инфекција семена и хипокотила, као и њихов раст. На основу ових параметара најбољи резултат је добијен након комбиноване употребе 5% варикине и суве стерилизације семена на 45°C.

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Vesna Krnjaja[1], *Jelena Lević*[2], *M. Ivanović*[3], *Zorica Tomić*[1]

[1] Institute for Animal Husbandry, Belgrade—Zemun, Serbia and Montenegro
[2] Maize Research Institute „Zemun Polje", Belgrade—Zemun, Serbia and Montenegro
[3] Faculty of Agriculture, Belgrade—Zemun, Serbia and Montenegro

VIRULENCE OF *FUSARIUM* SPECIES TO ALFALFA SEEDLINGS*

ABSTRACT: In *in vitro* conditions, virulence of 91 isolates of species *Fusarium genus* (*F. oxysporum, F. solani, F. acuminatum, F. equiseti, F. arthrosporioides, F. proliferatum, F. avenaceum, F. semitectum, F. tricinctum, F. sporotrichioides* and *F. graminearum*) towards alfalfa seedlings was investigated. Isolates of investigated species originated from diseased alfalfa plants collected on four locations in Serbia based on symptoms of wilting caused by fusarium and root rotting. Pathogenicity and virulence of investigated isolates of *Fusarium* spp. were determined by visual evaluation of inoculated seedlings of cultivar K28 in laboratory conditions. All isolated of investigated species had pathogenic effect on alfalfa seedlings, which expressed symptoms such as necrosis of root, moist rotting and „melting of seedlings". Colour of necrotic root tissue varied from light brown, brown, lipstick red to explicit black, depending on the *Fusarium* species. Strong virulence was established in 48 isolates, medium virulence in 31 and weak virulence in 12 isolates.

KEY WORDS: alfalfa (*Medicago sativa* L.), seedlings, *Fusarium* spp., virulence

INTRODUCTION

Longevity of alfalfa crops is conditioned by condition of root system, primarily in the root neck and small roots in the most active zone of the root. Therefore, rotting of root and root neck, since frequent in case of alfalfa, is one of the most important factors which reduces the longevity of alfalfa crops, yield and quality of alfalfa. *Fusarium* species isolated from diseased alfalfa roots, especially root neck, were more frequent than any other type of fungus. Except type of rot, symptoms of disease are manifested in form of chlorisis of leaves and lower plants.

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Fusarium oxysporum Schlect, *F. solani* (Mart.) Appel & Wollenw. Emend. Snyder & Hansen and *F. roseum* Lk. ex Fr. Emend. Snyd. & Hans. are species constantly isolated from alfalfa root (O'Rourke and Millar, 1966; Graham et al., 1979). According to data from literature regarding the etiology of root rot, other *Fusarium* species are also important, such as *F. avenaceum* (Fr.) Sacc., *F. arthrosporioides* Sherb., *F. culmorum* (W. G. Smith) Sacc., *F. scirpi* Lamb, et Fautr. var. *acuminatum, F. poae* (Peck) Wr., *F. sambucinum* Fuckel and *F. tricinctum* (Corda) Sacc. (Erwin, 1954; Chi et al., 1964; Nedelnik, 1988; Hwang et al., 1989).

On territory of Serbia, from alfalfa plants demonstrating symptoms of wilting and rotting of root and root neck, most frequently isolated were numerous *Fusarium* species (Milijić et al., 1984, 1986, Vico et al., 1996; Krnjaja and Ivanović, 2001; Krnjaja et al., 2002). Damage caused by nematodes (*Pratylenchus penetrans* Cobb) enables more intensive development of *Fusarium* wilt (*Fusarium oxysporum* var. *medicaginis*) on alfalfa root (Grujičić et al., 1984). *Fusarium* species were isolated also from alfalfa seed (Krnjaja et al., 2003), which could be source of further spreading of pathogens on vegetative parts of the plant and cause problem in establishing of alfalfa crops.

Considering how frequent incidences of fuzariozing wilting and root rotting are in alfalfa crops on the territory of Serbia as well as great number of isolated *Fusarium* species, objective of this research was to investigate virulence of different types of *Fusarium* species to alfalfa seedlings.

MATERIAL AND METHODS

Applying standard phyto-pathological methods, isolates of *Fusarium* spp. were separated from alfalfa plants, collected in the vicinity of Belgrade (Zemun, Padinska Skela), Novi Sad (Rimski Šančevi) and Kruševac, with symptoms of *Fusarium* wilt and root rot. According to morphological characteristics described by Nelson et al. (1983) and Burgess et al. (1994), investigated isolates belong to following species: *F. oxysporum, F. solani, F. acuminatum, F. equiseti, F. arthrosporioides, F. proliferatum, F. avenaceum, F. semitectum, F. tricinctum, F. sporotrichioides* and *F. graminearum*.

In *in vitro* conditions, inoculation of seedlings of cultivar K28 was carried out according to method described by Chi et al. (1964). Alfalfa seed was disinfected in 95% ethanol for 10 seconds, subsequently in 7% sodium hypo chlorite (NaOCl) for 10 minutes, rinsed in sterile water and dried on room temperature. Section of the colony of investigated isolates 4—5 mm^2 in diameter and five days old was placed in centre of Petri dish with 1,7% potato dextrose agar. Around the section of colony, on distance of 2 cm in diameter, 15 seeds of alfalfa were placed. Petri dishes were incubated on room temperature. After two days, primary roots were placed so that their tips were touching the rim of the fungus colony in the centre of Petri dish.

After 10 day incubation, degree of pathogenicity (virulence) of isolates was evaluated by visual inspection of necrotic areas according to following scale: 0 = no virulence (no necrotic areas on the root), 1 = weak virulence

(necrosis on the tip of the root), 2 = medium virulence (root and low part of the stem — stem butt, but necrosis or fungus mycelium didn't spread on leaves and upper section of stem) and 3 = strong virulence (necrosis or fungus mycelium have spread entirely over root, stems and leaves, and in some cases even „melting" of seedlings occurred).

RESULTS OF INVESTIGATION

By inoculation of alfalfa seedlings in laboratory conditions it was established that all 11 isolates of *Fusarium* species were pathogenic. Two days subsequent to contact between root and fungus colony necrosis appeared in all investigated isolates. Necrosis spread vertically and after 10 days of incubation isolates of strong virulence were completely spread over root, stems and leaves of seedlings, causing in some cases so called „melting" of seedlings (Fig. 1).

Necrotic tissue of the root was rotten and decayed. In case of isolates which haven't caused spreading of necrosis further from the root, herbaceous parts of seedlings which weren't necrotic tore easily when pulled from disintegrated and softened root tissue. Colour of necrotic root parts was light brown, brown, red brown, and lipstick red to black (Fig. 1). Roots of control seedlings were without necrosis, healthy and with stabile structure.

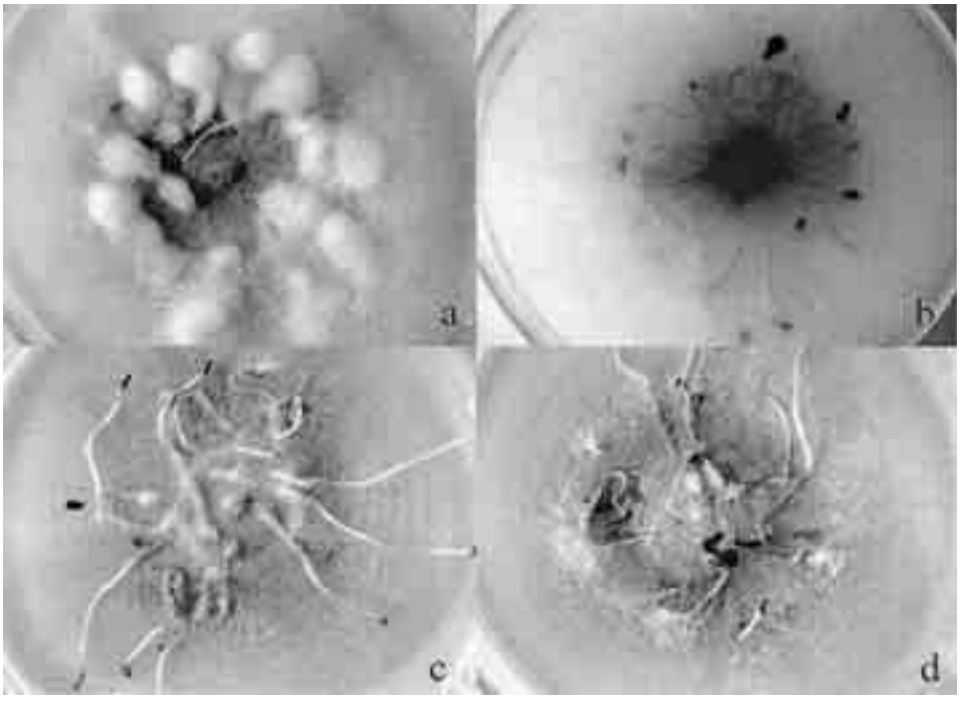

Figure 1. Appearance of necrotic alfalfa seedlings. „Melting" of seedlings in air (a) and substrate (b) section of the colony of the isolate LU32ZE; necrosis of root in the colonies of the isolates LU24ZE (c) and LU33ZE (d).

Among investigated isolates *Fusarium* spp. 48 demonstrated strong virulence, 31 isolates medium virulence, and weak virulence 12 isolates (tab. 1). Among investigated isolates of *F. oxysporum* 16 isolates demonstrated strong virulence (grade 3), 14 isolates demonstrated medium virulence (grade 2), and 4 isolates demonstrated weak virulence (grade 1). Among investigated isolates of *F. solani* 16 isolates demonstrated strong virulence, and one isolate demonstrated medium virulence. Seven isolates of *F. acuminatum* demonstrated strong, and two weak virulence. All investigated isolates of *F. equiseti* demonstrated weak virulence. Six isolates of *F. arthrosporioides* demonstrated medium virulence and one isolate strong virulence. All investigated isolates of *F. proliferatum* demonstrated strong virulence. One isolate of *F. avenaceum* demonstrated strong, and two isolates medium virulence. All investigated isolates of species *F. semitectum* and *F. sporotrichioides* demonstrated medium virulence. One isolate of *F. tricinctum* demonstrated strong, and one medium virulence. One investigated isolate of *F. graminearum* demonstrated strong virulence (tab. 1).

Table 1. Degree of pathogenicity (virulence*) of isolates of *Fusarium* species to alfalfa seedlings in *in vitro* conditions

Isolate	Virulence	Isolate	Virulence	Isolate	Virulence
		F. oxysporum			
LU4ZE	2	LU20ZE	3	LU6RS	2
LU5ZE	2	LU21ZE	1	LU10RS	2
LU6ZE	2	LU22ZE	2	LU22RS	2
LU7ZE	2	LU23ZE	2	LU3KS	3
LU8ZE	3	LU30ZE	2	LU5KS	3
LU9ZE	1	LU32ZE	3	LU15KS	3
LU10ZE	1	LU43ZE	3	LU16KS	3
LU11ZE	2	LU44ZE	3	LU17KS	3
LU12ZE	2	LU45ZE	3	LU1PS	3
LU14ZE	1	LU47ZE	3	LU4PS	3
LU16ZE	2	LU49ZE	3		
LU17ZE	2	LU1RS	3		
		F. solani			
LU24ZE	3	LU33ZE	3	LU40ZE	3
LU25ZE	3	LU34ZE	3	LU41ZE	3
LU26ZE	3	LU36ZE	3	LU42ZE	3
LU28ZE	3	LU37ZE	3	LU46ZE	3
LU29ZE	3	LU38ZE	3	LU48ZE	2
LU31ZE	3	LU39ZE	3		
		F. acuminatum			
LU6PS	3	LU10KS	3	LU16RS	3
LU4KS	2	LU7RS	3	LU19RS	3
LU9KS/1	2	LU8RS	3	LU24RS	3

F. equiseti					
LU2KS	1	LU3RS	1	LU25RS	1
LU6KS	1	LU5RS	1	LU26RS	1
LU8KS	1	LU12RS	1		
F. arthrosporioides				*F. proliferation*	
LU1ZE	3	LU17RS	2	LU2PS	3
LU2ZE	2	LU18RS	2	LU13KS	3
LU3PS	2			LU14KS	3
LU9KS/2	2			LU20RS	3
LU2RS	2			LU21RS	3
F. avenaceum		*F. semitectum*		*F. tricinctum*	
LU9RS	3	LU3ZE	2	LU7KS	2
LU27ZE	2	LU5PS	2	LU11KS	3
LU35ZE	2	LU13RS	2		
F. sporotrichioides		*F. graminearum*		Kontrola	
LU1KS	2	LU18ZE	3	—	0
LU23RS	3				

* 0 = no virulence, 1 = weak virulence, 2 = medium virulence, 3 = strong virulence

DISCUSSION

In the test for control of pathogenicity of *Fusarium* species to alfalfa seedlings pathogenicity of isolates of all investigated *Fusarium* spp. was established, as well as high sensitivity of alfalfa in pheno — stage of seedlings. Similar results were confirmed in previous investigations (W e i m e r, 1927, 1928, loc. cit. S c h m i t t e n n e r, 1964; C h i et al., 1964; H a n c o c k, 1983, 1985) when it was proved that *Fusarium* species can infect alfalfa seedlings. W e i m e r (1927, loc. cit. S c h m i t t e n n e r, 1964) has established that *Fusarium* spp. and *Rhizoctonia* spp., isolated from rotten root neck and root of alfalfa cause moist rotting of seedlings. *F. oxysporum* f. sp. *medicaginis* is also pathogenic to alfalfa seedlings (W e i m e r, 1928 loc. cit. S c h m i t t e n n e r, 1964). Isloates of *Rhizoctonia* spp. and *F. oxysporum* f. sp. *medicaginis* originating from alfalfa have demonstrated strong pathogenicity not only to alfalfa seedlings but also to seedlings of bird's foot trefoil, red and white clover (V i c o, 1997). Histological researches have shown that penetration and further development of *F. avenaceum, F. oxysporum* and *F. solani* are similar in case of alfalfa and red clover seedlings (C h i et al., 1964). Results obtained by these authors indicate that all three *Fusarium* species have penetrated into uninjured epidermal root cells, seed coat and cotyledons by direct penetration without formation of apresoria. Penetration was intercellular and intracellular. Most frequently, pathogens penetrated the meristematic tissue, although regions of cell magnification and differentiation were also affected. Pathogens colonize completely cortex of the alfalfa root. All three species colonize xylem, and *F. solani* is most limited when developing in epidermal and cortical tissu-

es. Tips of roots are affected by pathogens in two-day old seedlings. Seed coat was colonized quickly by all three fungus species. Lot of hyphae were found in cotyledons, leaf primordia and young stems. No difference was established between plants which became diseased naturally and artificially inoculated plants in regard to development of fungus (C h i et al., 1964).

CONCLUSION

Investigations of pathogenicity and virulence of *Fusarium* species *in vitro* have lead to following conclusions:

— *F. oxysporum, F. solani, F. acuminatum, F. equiseti, F. arthrosporioides, F. proliferatum, F. avenaceum, F. semitectum, F. tricinctum, F. sporotrichioides* and *F. graminearum* are pathogen to seedlings of K28 alfalfa;

— Main symptoms of disease are change of colour from brown to black depending on the investigated species, necrosis of root, moist rotting and „melting of seedlings";

— Most of the isolates demonstrated strong virulence (48) to medium virulence (31), and only 12 weak virulence, none of the isolates were no virulence;

— *F. solani* and *F. acuminatum* demonstrated mostly strong virulence, *F. arthrosporioides* medium virulence, whereas virulence of *F. oxysporum* varied from weak to strong;

— All isolated of *F. proliferatum* demonstrated strong virulence, and of *F. equiseti* weak virulence;

— Less present species on alfalfa root, such as *F. avenaceum, F. tricinctum* and *F. graminearum*, demonstrated medium to strong virulence, and *F. semitectum* and *F. sporotrichioides* medium virulence.

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ВИРУЛЕНТНОСТ ВРСТА РОДА *FUSARIUM* ПРЕМА КЛИЈАНЦИМА ЛУЦЕРКЕ

Весна Крњаја[1], Јелена Левић[2], М. Ивановић[3], Зорица Томић[1]
[1] Институт за сточарство, Београд—Земун, Србија и Црна Гора
[2] Институт за кукуруз „Земун Поље", Београд—Земун, Србија и Црна Гора
[3] Пољопривредни факултет, Београд—Земун, Србија и Црна Гора

Резиме

У *in vitro* условима проучена је вирулентност 91-ог изолата врста рода *Fusarium* (*F. oxysporum, F. solani, F. acuminatum, F. equiseti, F. arthrosporioides, F. proliferatum, F. avenaceum, F. semitectum, F. tricinctum, F. sporotrichioides* и *F. graminearum*) према клијанцима луцерке. Изолати испитиваних врста пореклом су из оболелих биљака луцерке које су прикупљене из четири локалитета у Србији на основу симптома фузариозног увенућа и трулежи корена. Патогеност и вирулентност испитиваних изолата *Fusarium* spp. утврђени су визуелним оцењивањем инокулисаних клијанаца сорте К28 у лабораторијским условима. Сви изолати испитиваних врста патогени су према клијанцима луцерке, који су испољили симптоме у виду некрозе корена, влажне трулежи и „топљења клијанаца". Боја некротираног ткива корена варира од светло смеђе, смеђе, црвено-смеђе, кармин црвене до изразито црне, зависно од врсте рода *Fusarium*. Јаку вирулентност испољило је 48 изолата, средњу 31 изолат, а слабу 12 изолата.

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Dobrila Jakić-Dimić[1], *Svetlana Jeremić*[2], *Ksenija Nešić*[3], *V. Radosavljević*[4]

[1] Viši naučni saradnik
[2] Viši naučni saradnik
[3] Istraživač saradnik
[4] DVM-Naučni institut za veterinarstvo Srbije, Vojvode Toze 14, 11000 Beograd, Srbija i Crna Gora

THE INFLUENCE OF MYCOTOXINS IN FOOD ON FISH HEALTH STATUS*

ABSTRACT: In our country, there is present extensive, semi-intensive and intensive growing of cyprinid fish species. The quality of food is an essential prerequisite for obtaining optimal production results in fish production.

Fish food is being produced as a complete pellet meal, and raw materials used are of plant, animal, mineral and vitamin origin. Out of plant feed, the most commonly used ones are corn, wheat, barley, oats, soy and others. By applying additional carbohydrate food, energetic needs of an organism are being met.

In this paper, we presented the results of hygienic safety of carbohydrate feed (corn, wheat, barley) investigated in the laboratory of Veterinary Research Institute of Serbia in Belgrade within regular control, or with the aim of establishing the causes of disturbance of health status and decreased production results in the pond.

During 2004 we performed microbiology and mycotoxicology investigations of the total of 43 samples, namely: 31 corn samples, 8 barley samples and 4 wheat samples.

The obtained results point at a high level of mould contamination (*Aspergillus, Penicillium, Fusarium, and Rhizopus*) and the presence of their secondary mycotoxin metabolites (aflatoxin, ochratoxin, trichothecenes and zearalenone) in feed.

KEY WORDS: cyprinid fish species, food of plant, animal, mineral and vitamin origin, hygienic safety, microbiology and mycotoxicology investigations, mould contamination.

INTRODUCTION

Nutritive requirements for growth, reproduction and all physiology functions of fish are similar to other animals. Everyday intake of proteins, minerals, vitamins, growth factors and energy is necessary.

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Cereals as energy and leguminose as protein feedstuffs are the main part of feed (up to 90%) for all kinds and categories of fish.

Usage of complete mixtures without appropriate hygiene qualities leads to decrease of productive results and progressive lowering of health of fish. Cereals — carbohydrate feedstuffs are a good medium for growth of number of fungi. In adequate conditions they multiply and their metabolites cause changes in feed quality. Bad feed can induce consequences on health and productive results of fish (L e l l e s, W. et al., 2000).

Cereals become contaminated with fungi in the field, during processing, storage, transport and usage. Some fungi cause detrimental effects in feed, either by decomposition of its components or by producing harmful metabolites (R a j i ć, I., 1993).

Mycotoxins are secondary metabolites of fungi which are formed during consecutive serial enzyme reactions over several biochemically simple intermediary products, from prime metabolism, acetates, mevalonates, malonite and some aminoacids (M a š i ć, Z., 1993; M a š i ć et al., 2002).

Their chemical characteristics and biology activities are very wide and able to cause different pathology and pathohistology changes in fish.

Mycotoxins are important contaminants of environment. They enter organisms by ingestion, but also by inhalation according to WHO report in 1979. Quite small amounts of these substances can harm health. High concentrations of mycotoxins are able to provoke acute disorders and can cause cancerogenic, mutagenic and teratogenic effects.

It is proved that mycotoxins production depends on:

— presence of toxin producing fungi
— convenience of substrate for fungal growth
— environmental conditions for fungal growth.

Fungi will produce mycotoxins only if these conditions are complied. Toxin productive strains of fungi are able to produce more than one toxin and also one toxin can be produced by different strains of fungi. But, the presence of fungal strains which are potential toxin producers in feed is indication of possible presence of mycotoxins.

High moisture (20—25%) is important factor for fungal growth in the field and in the raw plant material. Storage fungi are capable to rise in substrates which contain 12—18% of moisture. Contamination of feed with fungi, their growth and mycotoxin production during harvesting, transport, storage and mixing of agricultural products are under the influence of several factors: moisture, temperature, aeration and presence of other microorganisms.

Recent studies show that growth and toxin production of *Aspergillus* (aflatoxin) and *Penicillium* (ochratoxin A, patulin) are under the influence of maximal and minimal water activity and temperature values.

Water activity and temperature are specific for every fungal kind growth and very important for mycotoxin production, e. g. aflatoxin B_1 can be produced in such conditions that water activity and temperature are close to minimal for growth of other microorganisms. Patulin, penicillium acid and ochratoxin A are produced at lower water activity and temperature values, mainly mini-

mal temperature for *Aspergillus* growth and toxin production is higher than for *Penicillium*.

The most important mycotoxicoses in fish are caused by aflatoxins, ochratoxins, zearalenone and trichotecenes. Also recently, due to new methods which are very reliable in quantitative determination of fungi, a great improvement can be noticed in struggle with fungi as fish feed contaminants (Robinson, 1993; Lim and Dominy, 1990).

Experience in analysing feed, which is usually in use in fish feeding, has brought a need of detailed investigation to ensure on time diseases prevention. The aim of this work was testing of mycotoxins presence usually found in fish feed.

MATERIALS AND METHODS

During 2004. microbiological and mycotoxicological investigations of total of 43 samples (31 samples of corn, 8 samples of barley and 4 samples of wheat) were done. Samples of carbohydrate feedstuffs from fish farms from allover Republic of Serbia were sent for analyses to laboratories of the Scientific Institute of Veterinary Medicine of Serbia in Belgrade partly as routine control, but mostly in case of suspicion about feed quality and its possible connection with decrease of productive results and health disturbances.

For fungi determination a standard mycology method is implemented. For mycotoxicology examination of aflatoxin, ochratoxin and zearalenone presence in samples ELISA quantitative method, which is based on antigen-antibody reaction, is used. In the wells of microtiter strips, according to the commercial kit guide (R-Biopharm, Deutschland: Aflatoxin total, Ochratoxin A, Zearalenon zearalenone), standards and prepared samples are added. As the reaction has to become visible addition of enzyme and chromogen gives a blue coloured product which changes into yellow after the addition of the stop reagent. Finally, the measurement is made photometrically at 450 nm and the absorption is inversely proportional to the toxin concentration in the sample. Results are interpreted and compared to Rulebook of maximal amounts of detrimental substances in feed (Sl. list 2/90).

RESULTS AND DISCUSSION

According to mycology and mycotoxicology examinations of carbohydrate feedstuff samples at the Department for animal nutrition in the Scientific Institute of Veterinary Medicine of Serbia in Belgrade high contamination results are obtained. Almost 100% of samples were infested with fungi (Table 1 and 2).

The most common mycotoxins are zearalenone, ochratoxin A and aflatoxin B_1 and the highest degree of contamination is registered in corn samples (Table 3).

Table 1. Examined feedingstuffs

feedingstuffs	N° of samples	N° of samples contaminated with fungi	% of contaminated samples
corn	31	31	100
wheat	4	3	75
barley	8	8	100
total	43	42	97,67

Table 2. Commonly isolated fungi

	N° of samples	N° of infested samples	isolated fungi
corn	31	31	*Penicillium, Mucor, Fusarium, Aspergillus, Rhisopus*
wheat	4	3	*Penicillium, Mucor, Fusarium, Aspergillus, Rhisopus*
barley	8	8	*Penicillium, Mucor, Fusarium, Aspergillus*

Table 3. Mycotoxin content in feedstuffs, [mg/kg]

	corn	wheat	barley	x ± Sd	iv
zearalenone	5,30	2,06	2,00	3,12	0,80—5,33
ochratoxin A	0,20	0,22	0,30	0,24	0,16—0,35
aflatoxin B1	0,04	0,005	0,02	0,02	0,00—0,05

Different organs in fish organism are sensitive to different mycotoxins. According to the characteristics mycotoxicoses are similar to diseases caused by other pathogens or nutritive deficiency and disbalance.

Degree of changes caused by mycotoxins depends on type and the amount of mycotoxins in feed, also on exposure duration, age and species of fish. Mycotoxins induce several disorders in fish organism: biochemical, functional, morphological and in more severe cases mortality. Biochemical alterations and metabolism disturbance lead to changes in nutrient resorption and primary brings to cell and organ alterations.

Toxic effects of certain mycotoxins differ according to age and species of fish. Younger fish are more sensitive.

Aflatoxicoses of salmonids occurs as a consequence of presence of fungi *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus versicolor* and *Penicillium.* The major products of these molds are aflatoxins B_1, B_2, G_1, G_2. Other intermediates in the biosintetic pathway of this molds, namely, versicolorin A and sterigmatocystin, can also appear in contaminated feeds. Aflatoxins are hepatotoxins, also well known as carcinogens.

Aflatoxin penatrates into the cells, binds to the DNA molecules, inhibits polymerase enzyme and RNA synthesis, that leads to cell changes. In liver aflatoxin B_1 in presence of enzyme transformes into several metabolites. Aflatoxin metabolites are thought to be the most mutagenic agents which accumulate in hepatic tissue.

First reports about aflatoxin toxicity are made by Halver (1965, 1967) and Bauer et al. (1969). They have noted that aflatoxins are powerful carcinogens in rainbow trout. The hepatocarcinomata usually reach a clinical level after 4—6 month of feeding of the contaminated meal. The amount of contamination can be very small, as little as 0,1 ppb in the total diet.

Pathomorfological changes depend on species and age of fish, as well as on amount of mycotoxins in feed. Aflatoxicoses can manifest in acute or chronic form.

When fed experimentally at high levels, 80 ppb or more, the toxin produces an acute toxic syndrome, severe or even massive focal hepatic necroses, and branchial edema, as well as generalized punctate hemorrhage (Ashley, 1970).

Acute form manifests usually after 12 hours and chronic form after prolonged period of contaminated feed intake. Pathomorphological alterations are mainly located in liver as anaemia (pale liver) with focal hepatic necrosis and hemorrhage and renal inflammatory changes. Chronic form, in older fish, is characterised with invasive malignant trabecular hepatocarcinomata, very obvious because of the focal, darker zones of malignancy.

Ochratoxins (A, B, C, α, β) are products of isocumarin binded to L β-phenilalanine.

Ochratoxin A, the most toxic of the metabolites, produced by *Aspergillus ochraceus*, is a potential fish toxin since it occurs as a natural contaminant of corn and wheat (Shotwell i sar., 1969; Scott i sar, 1970). Presence of aflatoxin in fish feed indicates its possible presence in fish tissues. It causes degenerative changes in liver and necrosis of the proximal tubules, hematopoietic tissue, and glomeruli of the kidney. Ochratoxin A was found to be letal, with an LD_{50} of 4,67 mg/kg.

Ochratoxin B, the dechlorinated form of ochratoxin A, was nonlethal at doses up to 66,7 mg/kg, but the high dose caused some histological changes in the kidneys and liver similar to those caused by low doses of ochratoxin A.

Trichotecenes are secondary metabolites of several fungal genera. Mostly, they are produced by *Fusarium species, Trihotecium, Myrhotecium*, with 18 species in total, and around 100 compounds are chemically described. The most important natural trichotecens which cause health disturbances are DON--deoxynivalenol or vomitoxin and T-2 toxin.

Vomitoxin is one of the naturally occuring trichotecene mycotoxins produced by genus *Fusarium* that grow on various cereals grains such as corn, barley, and wheat. Because wheat and the corn products are used frequently in cyprinid diets, vomitoxin is a potential problem for carp culture. Fish receiving low levels (1—12,9 μg/g) of vomitoxin are the diets but demonstrated reduced growth and feed efficiency, neither clinical signs nor mortalities were observed during the 8-weeks study (Woodward et al., 1983).

T-2 toxin is another trichotecene mycotoxin produced by *Fusarium* species growing on cereal grains. Its detrimental influence is manifested in dosed above 2.5 mg/kg as depressed growth, efficiency of feed use, hematocrit, blood hemoglobin concentration, and feed acceptance. A single acute oral dose (6.5 mg/kg body wt) given to rainbow trout fingerlings caused extensive shed-

ding of the intestinal mucosa, severe edema in body cavities, and eventual death (M a r a s a s, 1967). Long-term (12 month) ingestion of low doses (200—400 μg/g feed) in older fish had no apparent adverse effect and actually promoted better growth than the control.

CONCLUSION

Losses in aquaculture caused by mycotoxins in feed can be significant. Direct loss is a consequence of increased mortality and indirect loss is result of decrease of production and occuring of secondary diseases.

Preventive measures consist of agrotechnical and agrochemical operations which are implemented to inhibit fungal growth in fish feed.

Decrease of grain damaging and moisture, on time application of fungicides and warehouse aeration appeared to be effective in struggle with molds in fish feed. The safest way to avoid problems is not to use mycotoxin contaminated feed in fish nutrition at all.

The most common mycotoxins are described. However, this does not imply that other may not be important. As new feed ingredients are indentified and incorporated in fish diets their mold contaminants will need to be identified and tested for possible deletrious effectious. Whenever general pathological symptoms occur in hatchery fish, the role of mycotoxin should not be overlooked. It is likely that the toxicities of several mold metabolites new to fish remain to be discovered and researchers are encouraged to test the toxicities of potentially important mycotoxins on various species of fish.

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УТИЦАЈ МИКОТОКСИНА НА ЗДРАВЉЕ РИБЕ

Добрила Јакић-Димић[1], Светлана Јеремић[2],
Ксенија Нешић[3], В. Радосављевић[4]
[1] Виши научни сарадник, [2] Виши научни сарадник, [3] Истраживач сарадник
[4] ДВМ-Научни институт за ветеринарство Србије, Војводе Тозе 14,
11000 Београд, Србија и Црна Гора

Резиме

У нашој земљи је заступљено екстензивно, полуинтензивно и интензивно гајење ципринидних врста риба. Квалитет хране је битан предуслов за постизање оптималних производних резултата у рибарству.

Храна за рибе производи се као пелетиран комплетан оброк, а сировине које се употребљавају су биљног, анималног, минералног и витаминског порекла. Од биљних хранива најчешће се употребљавају кукуруз, пшеница, јечам, овас, соја и др. Додатном угљенохидратном храном се задовољавају енергетске потребе организма.

У раду су приказани резултати хигијенске исправности угљенохидратних хранива (кукуруз, пшеница, јечам) испитиваних у лабораторији Научног института за ветеринарство Србије у Београду у оквиру редовне контроле или у циљу утврђивања узрока поремећаја здравственог стања и лошијих производних резултата на рибњаку.

У току 2004. године извршена су микробиолошка и микотоксиколошка испитивања укупно 43 узорка: 31 узорак кукуруза, 8 узорака јечма и 4 узорка пшенице.

Добијени резултати указују на висок степен контаминације гљивицама (*Aspergillus, Penicillium, Fusarium, Rhizopus*) и на присуство њихових секундарних метаболита микотоксина (афлатоксин, охратоксин, трихтецени, зеараленон) у хранивима.

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Gordana R. Dimić, Željko M. Maletić, Sunčica D. Kocić-Tanackov

Faculty of Technology, Bulevar Cara Lazara 1,
21000 Novi Sad, Serbia and Montenegro

XEROTOLERANT MYCOPOPULATIONS AND MYCOTOXINS IN MUESLI COMPONENTS*

ABSTRACT: The presence of fungi was investigated in 22 samples of different dried plant origin products used for the preparation of muesli (grain products, dried fruit, nuts, oilseeds), using three media. The determined contamination levels were between 0,6% (grain products) and 46,4% (raisins).

The xerotolerant *Aspergillus, Penicillium* and *Eurotium* species, mostly toxigenic, and fungi from *Rhizopus* genera, were the most frequent in the investigated samples.

Aflatoxin B1 (AB1) was not detected in any sample, while aflatoxin G1 (AG1) was found in one almond sample (0,14 μg/kg). Two almond samples were contaminated with ochratoxin A (OA), 8,00 and 16,00 μg/kg, and one sunflowerseed sample had it in traces. Zearalenone was found in two sunflowerseed samples (120,00 and 200,00 μg/kg).

KEY WORDS: contamination, mycotoxins, fungi, dried fruit products

INTRODUCTION

Muesli are the mixtures of different grain products in combination with dried fruit and nuts, oilseeds and other components. These products are customary present in vegetarian and macrobiotic nutrition. This type of foodstuff is also very often used in nutrition of other categories of population, with the aim to decrease obesity and other health problems, as well as the need for proper and balanced intake of nutrients. They are recommended as a biologically valuable meal and as the source of fibres.

However, the microbiological contamination of dried plant origin products is a very serious problem from the standpoint of both hygienic and health aspect. The grains and fruit are contaminated by different fungi species during the vegetation period, and some of them may produce toxic metaboli-

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tes. The possibility of subsequent contamination with fungi and mycotoxins is increasing during processing and further handling.

The xerotolerant (xerophylic) mycopopulation is regularly present in storehouses of dried products. The representatives of this group are some *Aspergillus* and *Penicillium* species, *Emericella, Eurotium, Paecilomyces, Wallernia, Xeromyces* and others (Pitt, 1975; Pitt and Hocking, 1985; Beuchat and Hocking, 1990).

This work includes the investigations of the presence of fungi in individual components used for the preparation of muesli, the presence of xerophilic populations and the most important mycotoxins.

MATERIALS AND METHODS

22 samples of dried plant products — components of muesli — were included in the investigations: grain flakes — wheat, oat, rye, barley, and corn (5 samples), raisins (5 samples), almond (3 samples), hazelnut (3 samples), sunflowerseed (3 samples) and naked pumpkinseed (3 samples).

Three mycologic media were used for the isolation of fungi and determination of total count: a) Sabouraud maltose agar- common medium; b) Czapek yeast extract agar with 20% of saccharose (CY20S): K_2HPO_4 — 1 g, Czapek concentrate — 10 ml, yeast extract — 5 g, saccharose — 200 g, agar — 15 g, distilled water — 1 l; c) Malt extract yeast extract 20% glucose agar (MY20G): malt extract — 10 g; yeast extract — 2,5 g, glucose — 200 g, agar — 10 g, distilled water up to 800 g.

The standard Koch's method was used as the isolation technic. The inoculated Petri dishes (in duplicate) were incubated for 7 days at 25°C.

The idenfitication and determination of fungal genera and species were carried out according to Ellis (1971) and Hocking (1985) and Samson and van Reenen-Hoekstr (1988).

The determination of toxic fungi metabolites, aflatoxins B1 (AB12) and G1 (AG1), ochratoxin A (OA) and zearalenone (ZEA) was performed using the multimycotoxinic method of Balzer et al. (1978). The toxins were determined by thin-layer chromatography (TLC). The determined toxins were quantified by visual comparison of fluorescence intensity of sample spots with the corresponding referent standard.

RESULTS AND DISCUSSION

Investigating the contamination with fungi of six groups of components used for the preparation of muesli (Figure 1), it was found that their occurrence was especially expressed in raisins, 46,4%. To lesser extent were contaminated the naked pumpkinseed (24,1%), almond (17,0%) and sunflowerseed (10,7%), while the contamination of grain flakes is practically negligable (0,6%).

The results of mycological investigations (Figure 2) showed that xerophylic micropopulations are the most frequent in dried vegetable products. Species from *Aspergillus* genus were isolated from 81,8%, *Penicillium* from 59,1% and *Eurotium* from 31,8% of samples. The presence of *Paecilomyces* genus was somewhat less (22,7%). The occurrence frequency of non-xerophilic fungi was: *Rhizopus* 45,4%, *Cladosporium* 27,3%, *Syncephalastrum* 22,7%, *Alternaria* 18,2%, *Fusarium* 13,6%, *Mucor* and *Trichoderma* 9,1%, *Aureobasidium, Moniliella* and *Scopulariopsis* 4,5%.

This second group of significantly more numerous genera is usually more active before drying and in this kind of foodstuff, which

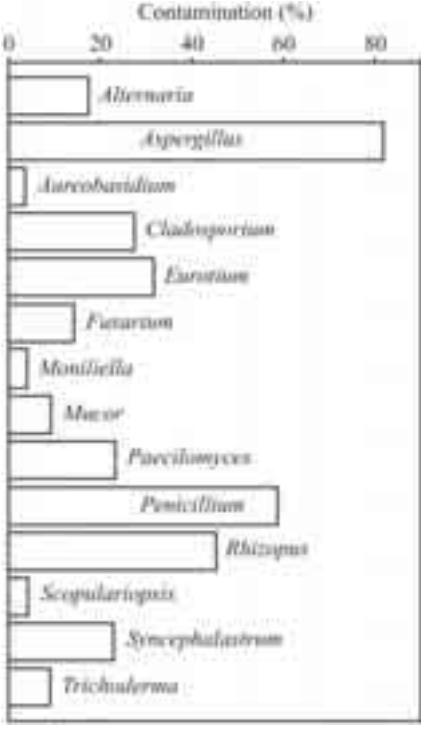

Figure 2. Presence of fungi genus

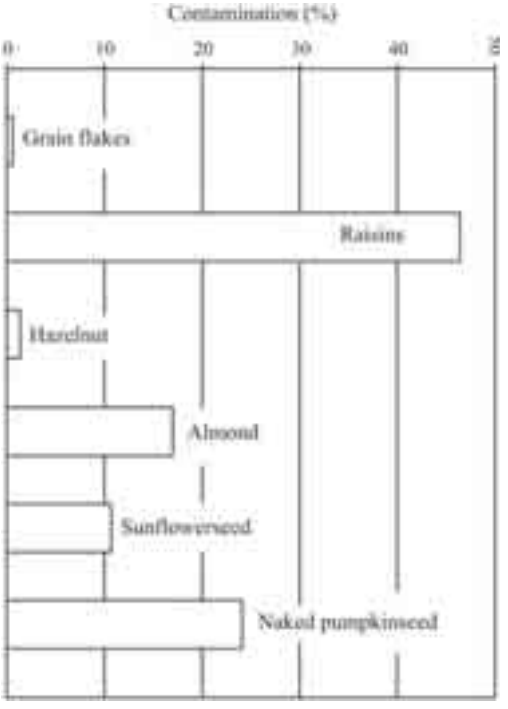

Figure 1. Contamination of muesli components by fungi

is insufficiently dry, however, all genera are not equally significant. *Fusarium* and *Alternaria* genera should be separated, as their representatives are known to synthesize different toxic metabolites (G o t o, 1990; V i n a s et al., 1992), when the substrate moisture content is above 20% (B u l l e r m a n et al., 1984). However, due to the ability of xerophylic fungi to grow under reduced moisture, they are significantly more difficult to control. Their growth depends on the a_w range from 0,80 to 0,61 (extreme xerophylic *Xeromyces bisporus, Chrisosporium fastidum* etc.) (B e u c h a t and H o c k i n g, 1990).

The xerophylic fungi isolated from muesli components are in the group of imperfect forms (Fungi imperfecti), or are the perfect forms of *Aspergillus* genus (*Eurotium*). Their presence is presented

in Table 1. Twenty one (21) xerophylic species were determined, representing 52,5% of total number of isolated ones (40). *A. niger* is dominant between them, and it's presence was found (registered) in raisin, almond, hazelnut, sunflowerseed and naked pumpkeen seed, followed by *A. flavus* and *P. aurantiogriseum*. Comparing the individual components it can be concluded that almond, hazelnut and sunflowerseed were contaminated with highest number of fungi species.

A. niger and *A. flavus* were found in 68,6 and 36,4% of samples, respectively, pointing to the domination of *Aspergillus* spp. in dried products. *Eurotium herbariorum* (22,7%) was the most frequent fungus of *Eurotium* genera. The same frequency was noted in *Penicillium aurantiogriseum* and *Paecilomyces variotii.* The ratio of *A. terreus, P. chrysogenum* and *P. glabrum* was also significant. The deterioration of walnut, dried fruit and different seeds may be connected with species isolated during these mycologic investigations (Pitt and Hocking, 1985; Abdelgavad and Zohri, 1993; Weidenborner and Kunz, 1994).

Table 1. Xerophilic fungi isolated from muesli components

Mould species	Grain flakes	Raisins	Almond	Hazelnut	Sun-flower-seed	Naked pumpkin-seed	Presence (%)
Aspergillus candidus						+	4,5
A. Flavus	+		+	+		+	36,4
A. niger		+	+	+	+	+	63,6
A. ochraceus					+		4,5
A. penicilloides					+		4,5
A. tamarii			+				4,5
A. terreus			+	+			18,2
A. versicolor	+			+			9,1
A. wentii					+		9,1
Eurotium amstelodami					+		9,1
E. chevalieri			+				4,5
E. herbariorum	+			+	+		22,7
Paecilomyces variotii			+	+			22,7
Penicillium aurantiogriseum	+	+			+	+	22,7
P. brevi-compactum	+				+		9,1
P. chrysogenum				+	+		13,6
P. glabrum		+	+	+			13,6
P. implicatum			+				4,5
P. restrictum						+	4,5
P. rugulosum			+				4,5
P. spinulosum				+			4,5

Most of the isolated species produce different toxic metabolites, however, *A. flavus* (aflatoxins), *A. ochraceus, P. aurantiogriseum, P. chrysogenum* (ochra-

toxin A), *A. versicolor, Eurotium* spp. (sterigmatocistin) and *Paecilomyces variotii* (patulin) are the producers of the most important mycotoxins (Hacking and Rosser, 1981; Frisvad, 1988; Goto, 1990; Duraković and Duraković, 2003). These toxins produced as secondary metabolites, are potential mutagenes, teratogenes, cancerogenic and immunosuppressive agents.

The results of the investigations of contamination with aflatoxins (AB1 and AG1) and ochratoxin A (OA), including the investigation of zearalenone of *Fusarium* spp. are presented in Table 2.

Table 2. Mycotoxins in muesli components

Sample	Aflatoxins (μg · kg^{-1})		Ochratoxin A (μg · kg^{-1})	Zearalenone (μg · kg^{-1})
	AB1	AG1		
Grain flakes:				
Wheat	—	—	—	—
Rye	—	—	—	—
Oat	—	—	—	—
Barley	—	—	—	—
Corn	—	—	—	—
Raisins				
1	—	—	—	—
1	—	—	—	—
3	—	—	—	—
Almond				
1	—	—	16,00	—
2	—	—	8,00	—
3	—	0,14	—	—
Hazelnut				
1	—	—	—	—
2	—	—	—	—
3	—	—	—	—
Sunflowerseed				
1	—	—	—	—
2	—	—	—	120,00
3	—	—	—	200,00
Naked pumpkinseed				
1	—	—	—	—

The presence of AB1 was not stated in any muesli component, while one almond sample contained AG1 in concentration of 0,14 μg/kg. Two almond samples were contaminated with OA in detectable quantities, and one sunflowerseed sample in traces. The toxin level in one almond sample was 16,00 μg/kg, and according to our legislations the maximum allowed limit of OA is 10,00 μg/kg (Yugoslav Official Regiester SRJ 5, 1992). Zearalenone was found in two sunflower samples, in high concentrations, 120,00 and 200,00 μg/kg.

In contrast to aflatoxin and ochratoxin, zearalenon acts as estrogenic hormone affecting in the first place the genital tract of laboratory animals (Ožegović and Pepeljnjak, 1995).

Having in mind that contamination occurs during the vegetation period of plants, there is no absolute safety from contamination with mycotoxins, however, severe control, preventing the contamination with and growth of these microorganisms throughout the whole production and processing chain, to the consumers, may be the preventive activities in decreasing the risk of the exposure to alimentary diseases. This is a very important problem as toxins are cummulating in the organism, their effect is long-lasting and it is very difficult to remove them from the living organism.

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КСЕРОТОЛЕРАНТНЕ МИКОПОПУЛАЦИЈЕ И МИКОТОКСИНИ У КОМПОНЕНТАМА МУСЛИЈА

Гордана Р. Димић, Жељко М. Малетић, Сунчица Д. Коцић-Танацков
Технолошки факултет, Бул. Цара Лазара 1,
21000 Нови Сад, Србија и Црна Гора

Резиме

Укупно 22 узорка различитих сувих биљних производа за припрему муслија (производи од житарица, сушено воће, језграсто воће, семе уљарица) испитана су на присуство плесни, на три подлоге. Утврђени нивои контаминације били су између 0,6% (производи од житарица) и 46,4% (суво грожђе).

Ксеротолерантне *Aspergillus*, *Penicillium* и *Eurotium* врсте, највећим делом токсигене, заједно са плеснима рода *Rhizopus*, биле су најзаступљеније у испитиваним узорцима.

Од узорака анализираних на афлатоксине, афлатоксин Б1 (АБ1) није детектован, док се афлатоксин Г1 (АГ1) налазио у једном узорку бадема (0,14 μg/kg). Охратоксином А (ОА) била су контаминирана два узорка бадема (8,00 и 16,00 μg/kg) и један сунцокрета (у траговима). Код два узорка сунцокрета пронађен је зеараленон (120,00 и 200,00 μg/kg).

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Radmila V. Marković, Nebojša D. Jovanović, Dragan S. Šefer, Zlatan J. Sinovec

Radmila Marković, asistent-pripravnik; mr Nebojša Jovanović, asistent; dr Dragan Šefer, docent; dr Zlatan Sinovec, red. profesor; Fakultet veterinarske medicine, 11000 Beograd, Serbia and Montenegro

MOULD AND MYCOTOXIN CONTAMINATION OF PIG AND POULTRY FEED*

ABSTRACT: During ten-year period (1995—2004), a totat of 756 analyses of pig and poultry feed was performed. Standard methods were used for microbiological determination. Qualitative and quantitative analyze of mycotoxins was performed by TLC technique.

Feed for young categories contained from 100 to 3,400,000 CFU/g of feed. In 35.71% of all samples the detected amount was above acceptable levels. Feed for adult categories contained from 800 to 8,000,000 CFU/g of feed. In only 7.54% of samples this amount was over the tolerable level. Species determination revealed great heterogeneity, with the most common findings of *Penicillium* spp. (28.38%), *Aspregillus* spp. (26.37%), *Mucor* spp. (24.67%), *Fusarium* spp. (11.33%) and *Rhizopus* spp. (9.22%).

The amount and type of mycotoxin varied depending on the feed category as well as on year of detection, implicating a strong influence of climatic factors and average humidity of the specified year. In a total of 320 analyzed feeds for pigs and poultry the characteristic finding was a combined contamination with two or three mycotoxins.

In 161 samples of feed for young animals the presence of AFB1, F-2 and OTA was detected in 36, 161 and 161 samples, respectively, while in 33, 83 and 71 samples the detected amounts were above tolerable levels.

In 159 samples of feed for adult animals the presence of AFB1, F-2 and OTA was detected in 32, 159 and 159 samples, respectively, while in 31, 65 and 99 samples the detected amounts were above tolerable levels.

KEY WORDS: mould, mycotoxin, feed, pig, poultry

INTRODUCTION

Spoilage of the feed generally means the deviation of standard quality, which incorporates changes of organoleptic properties, nutritional value, as well as of hygienic properties. Spoiled feed could be potentially harmful, but

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not necessarily. Nevertheless, medical aspect of spoiled feed is expressed by the decrease of optimal animal performance, incidence of metabolic disorders, health disturbance, even with lethal outcome. Spoilage of feed is caused by chemical, physical, and biological effects. One of the most common is the presence of microorganisms. Because of their paired effects, via direct impact on nutritive value and/or by the production of mycotoxins, the presence of fungi and moulds in feed is considered as a very important issue in the determination of the overall feed quality. Thus, it could be interesting to evaluate the results of feed used in pig and poultry nutrition, analyzed in our laboratory, and to compare the results with the current legislative, as well as with our earlier findings (Sinovec et al., 1989; Šefer et al., 1989; Nedeljković et al., 1994; Šefer et al., 1994).

MATERIAL AND METHOD

During ten-year period (1995—2004), a total amount of 756 analyses of feed samples for all the categories of pigs and poultry was performed. Standard microbiological methods were used for the determination of microorganism presence. Mycotoxins were detected qualitatively and quantitatively by TLC chromatography (AOAC, 1980). All the results were statistically analyzed and compared with the maximal tolerable amounts stipulated by the current legislative (*Pravilnik*, 1990).

RESULTS

Feed for young categories contained from 100 to 3,400,000 CFU/g of feed. In 35.71% of all samples, the detected number was above maximum tolerable levels. Feed for adult categories contained from 800 to 8,000,000 CFU/g of feed. In only 7.54% of samples this amount was over the tolerable level. Regarding the category of animals, an interesting finding was the fact that feed for young animals is more often contaminated with higher amounts of moulds than allowed (Table 1).

Table 1. Mould contamination of fed for pigs and poultry

	Pigs			Poultry	
	Piglets	Fattening	Reproduction	Broilers	Layers
No. of samples	88	61	40	136	111
Over max. level, %	25	4.91	7.5	42.64	9

Species determination revealed great heterogeneity, with the most common findings of *Penicillium* spp. (28.38%), *Aspregillus* spp. (26.37%), *Mucor* spp. (24.67%), *Fusarium* spp. (11.33%) and *Rhizopus* spp. (9.22%).

The amount and type of mycotoxin varied depending on the feed category as well as on year of detection, implicating a strong influence of climatic

factors and average humidity of the specified year. Out of a total of 320 analyzed feeds for pigs and poultry the characteristic finding was a combined contamination with two (78.75%) or three (21.25%) mycotoxins.

Out of 161 samples of feed for young animals the presence of AFB1, F-2 and OTA was detected in 36, 161 and 161 samples, respectively, while in 33, 83 and 71 samples the detected amounts were above the tolerable levels.

Out of 159 samples of feed for adult animals the presence of AFB1, F-2 and OTA was detected in 32, 159 and 159 samples, respectively, while in 31, 65 and 99 samples the detected amounts were above the tolerable levels (Table 2).

Table 2. Feed samples contaminated with mycotoxins

		No of samples	$\bar{X}$		Sd	Over max. tolerable levels
Piglets	AFB1	23	0.05	±	0.02	13
	F-2	87	5.06	±	2.74	82
	OTA	87	0.27	±	0.23	87
Fattening	AFB1	8	0.06	±	0.04	8
	F-2	35	3.97	±	2.33	30
	OTA	35	0.31	±	0.14	29
Reproduction	AFB1	9	0.06	±	0.04	2
	F-2	36	5.25	±	3.20	34
	OTA	36	0.27	±	0.11	7
Broilers	AFB1	13	0.05	±	0.04	9
	F-2	74	5.14	±	2.69	0
	OTA	74	0.26	±	0.15	70
Layers	AFB1	16	0.04	±	0.03	16
	F-2	88	5.28	±	2.61	0
	OTA	88	0.23	±	0.12	41

DISCUSSION

Fungi and mould growth in feedstuffs is associated with the utilization of nutrients from the host, causing alteration in the nutritional content of the feedstuff. The germ of the grain is the main site for *Aspergillus* spp. development, thus the reduction of energy value due to fat utilization in contaminated grain should be expected. Having in mind that young animals are usually fed with high-energy feed, extra supplementation of oil due to decreased energy value will increase feed cost. On the other hand, formulating feed upon ingredient tables and not taking into account nutritional damage of specific loads could lead to undernutrition and decrease of animal performance. The consumption of mouldy feed caused growth depression in chicks (F r i t z et al., 1973). Not only fat, but also sugars are exposed to mould utilization, leading

to further decrease of nutritive value. The reduction of fat and carbon hydrates content due to mould growth and respiration caused a decrease of 5% in metabolical energy level (Bartov, 1982).

The changes in protein and amino acids content of mouldy grains are not well correlated, as it is the case with energy sources. Relative protein content in mouldy grains was slightly higher than in mould free ones (Cook, 1994), but probably due to a more intensive utilization of fat and carbohydrates compared to protein. Nevertheless, the total nitrogen content in moldy grains was decreased. Regarding the amino acids, it was concluded that the changes occurred in the amounts of some, especially essential ones. After moulding, a decrease in lysine (45%), arginine (50—54%), histidine (49.5%) and cysteine (74.5%), as well as an increase in methionine (34.5—54%) was observed (Kao and Robinson, 1972).

The development of moulds on stored feedstuffs may result from contamination in the field or during the storage. Isolated species in our case are mostly storage contaminators, implicating that the high number of contaminated feed is most probably the result of manipulative mistakes during storage of feedstuffs or feed. Inadequate environmental conditions in storage facilities, lack of control and other factors during feed production and manipulation could strongly contribute to the aggravation of this problem.

Adequate judgment of detected number of microorganisms in feed is even more demanding due to ambiguous legislative statements regarding tolerable amounts, especially when young and adult categories are in question. More precise criteria are needed concerning exact definitions of age and productive specificities of different animal species.

During unfavourable environmental conditions moulds are able to change metabolical pathways, which is consider to be a defensive mechanism. Secondary toxic metabolites, also known as mycotoxins, are produced by several genuses of moulds. The estimation is that approximately 25% of global grain production is contaminated by known mycotoxins (Davegowda et al., 1998), while even greater percentage could be contaminated with still unknown ones. Regarding those facts, the presence of mycotoxins in animal feed represents a great problem for animal production in our country. During 1999—2000 the presence of zearalenone, ochratoxin A, aflatoxin B_1 and T-2 toxin was found out in 72.3—74.5, 41.2—63.6, 20.1—21.65 and 29.7—45.1% of scrutinized feed samples (Bočarov-Stančić i sar., 2000). Moreover, laboratory data demonstrate that over 70% of feed samples are contaminated with two or more mycotoxins in the amounts above maximal approved limits (Šefer et al. 1994; Mašić et al., 2002).

Some of them have great nutritional, medical and economical importance in animal production (aflatoxins, ochratoxins, zearalenone, trichothecenes). They are mostly produced by *Aspregillus, Penicillium* and *Fusarium* species. Their impact on animal's health and performance are relatively well described in the literature. Functional and structural changes in target tissues, organs and systems (Humphreys, 1988; Willie and Morehouse, 1978) lead to deterioration of health with possible death, decrease in performance and significant economic losses.

Special attention should be paid to the impact of mycotoxins in human consumers. Of greatest concern in humans (Ellis et al., 1991) is AFB_1 implicated role in primary liver cancer in some geographical areas in Africa and Asia, where values of high dietary AFB_1 daily intake were encountered. It is difficult, however, to establish a possible casual role of AFB_1 in geographical areas where the incidence rate of primary liver cancer is very low, as in Europe and North America, and where the dietary AFB_1 daily intake is very low.

OTA is the main cause of an irreversible and fatal kidney disease (Balkan endemic nephropathy). It could be the result of the consumption of commodities directly contaminated with toxigenic strains of fungi, as well as by the consumption of meat of animals which have eaten OTA-contaminated feeds (Blunden et al., 1991).

F-2 toxin can produce oestrogenisation and pseudogravidity in women and it is related with prostate carcinoma. On the other hand, zearalenone derivates used as chemotherapeutics can benefit in menopause disturbances. Commercial application exists in some countries where α-zearalenone is used as growth stimulator in steer and lamb breeding (Hidy et al., 1977, US Food and Drug Administration 1980). Significance of zearalenone is connected with climate conditions in Balkan region that are optimal for *Fusarium* growth, as well as for the F-2 production. According to recent investigation zearalenone is the mycotoxin with the highest prevalence in feed for swine, with an increasing trend (Mašić et al., 2002), indicating that more than 75% of positive feed samples contained F-2 above the maximal tolerable levels.

CONCLUSION

The changes in the nutritive value that can occur after mould contamination of feed should be taken into consideration when feed efficiency is estimated regarding animal's performance. Although the major role in detrimental effects is contributed to the mycotoxins, it would be much precise if both factors — decrease of nutritive value and toxin impact — could be regarded together. The most important role of this interaction could be observed when low levels of mycotoxin are present.

Prevention and control of mould development should be one of the major tasks in the efforts to provide safe and adequate feed for domestic animals. Permanent monitoring is needed on all levels of production and storage, as well as the use of known methods to reduce mould contamination or toxin content in feedstuffs and feed. Improved legislative could further positively contribute to the better control and solution of mouldy feed problems. Preserved feed quality is the main condition in assurance of expected animal performance and good health, leading further to high quality of animal products, safe for human nutrition.

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КОНТАМИНАЦИЈА СМЕША ЗА ИСХРАНУ СВИЊА И ЖИВИНЕ ПЛЕСНИМА И МИКОТОКСИНИМА

Радмила В. Марковић, Небојша Д. Јовановић,
Драган С. Шефер, Златан Ј. Синовец
Факултет ветеринарске медицине,
11000 Београд, Србија и Црна Гора

Резиме

Током десетогодишњег периода (1995—2004) извршено је укупно 756 анализа узорака сточне хране намењених за исхрану живине и свиња. За микробиолошку анализу узорака коришћене су стандардне методе, а квалитативно и квантитативно испитивање наведених микотоксина извршено је ТЛЦ методом.

Смеше за младе животиње садржале су од 100 до 3.400.000 плесни/gr, при чему је чак 35.71% испитиваних узорака садржало недозвољен број плесни. Смеше за одрасле животиње садржале су од 800 до 8.000.000 плесни/gr, при чему је свега 7.54% испитиваних узорака садржало недозвољен број плесни. Врсте изолованих родова плесни показују велику хетерогеност, а најчешће су детектоване *Penicilium* spp. (28.38%), *Aspergillus* spp. (26.37%), *Mucor* spp. (24.67%), *Fusarium* spp. (11.33%) и *Rhisopus* spp. (9.22%).

Количина и врста микотоксина варира у односу на врсту смеше, као и у односу на поједине године, што може да се доведе у директну везу са климатским факторима, односно са годишњом влажношћу. Испитано је укупно 320 смеша за исхрану свиња и живине, а карактеристично је да је велик број смеша био контаминиран са два, односно три микотоксина.

Од укупно 161 узорка хране за младе животиње присуство АФБ1, Ф-2 и ОТА утврђено је у 36, 161 и 161 узорку, од чега је, истим редом, 33, 83 и 71 узорак садржао наведене микотоксине изнад дозвољене границе.

Од укупно 159 узорака хране за одрасле животиње присуство АФБ1, Ф-2 и ОТА утврђено је у 32, 159 и 159 узорака, од чега је, истим редом, 31, 65 и 99 узорака садржало наведене микотоксине изнад дозвољене границе.

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Leka Mandić[1], *Dragutin Đukić*[1], *Snežana Đorđevic*[2]

[1] Faculty of Agronomy, Cara Dušana 34, 32000 Čačak, Serbia and Montenegro
[2] Faculty of Agriculture, Nemanjina 6, 11081 Zemun, Serbia and Montenegro

SOIL FUNGI AS INDICATORS OF PESTICIDE SOIL POLLUTION*

ABSTRACT: Soil fungi, with their pronounced enzymic activity and high osmotic potential, represent a significant indicator of negative effects of different pesticides on the agroecosystem as a whole. In that respect, a trial was set up on the alluvium soil type with the aim to investigate the effect of different herbicides (Simazine, Napropamid, Paraquat), fungicides (Captan and Mancozeb) and insecticides (Fenitrothion and Dimethoate) on a number of soil fungi under apple trees.

The number of soil fungi was determined during four growing seasons by an indirect method of dilution addition on the Czapek agar.

The study results indicate that the fungi belong to the group of microorganisms that, after an initial sensible response to the presence of pesticides in the soil, very rapidly establish normal metabolism enabling them even to increase their number. The fungicides and insecticides applied were found to be particularly effective in that respect.

KEY WORDS: fungi, soil, pesticides, apple

INTRODUCTION

With the aim of obtaining high yields of agricultural crops, modern agricultural production demands use of different chemical compounds. According to the data obtained by Hajnis et al. (1979), 20% of crop farming production and almost 60% of fruit production are based on the use of chemical crop protection. Discontinuation of pesticide application, according to the FAO data, would decrease agricultural crops yield by 30—50% with the damage of about 75 billion dollars (Ejhler, 1986).

Besides an immediate desired effect, pesticides also have side-effects on biosphere, the extent of which is comparable to that of global ecological factors (Huston and Wagant, 1989). Soil microorganisms, particularly soil

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fungi, represent a biogeosphere component determining the level of their real toxic effect, since they take part in their detoxication and mineralization, using them as carbon and energy sources (Đukić and Mandić, 1998; Nunez et al., 2001). According to the data obtained by Bumpus and Tatarko (1994), the level of these processes depends on the soil-climatic conditions, oxidoreduction potential or, more precisely, on the secretion of enzymes of the lignin degradation system (of lignin peroxidase, manganese peroxidase, quinol oxidase etc.) — Stahl and Aust (1995).

On the other hand, high pesticide concentrations, decreased organic matter amount and soil moisture contribute to a decline in the number and activity of soil fungi (Kjoller and Rosendahl, 2000), impacting also the plant nutrition itself and change in soil structure and fertility (Bethlenfalvay and Shuepp, 1994).

The aim of the paper was to determine the effect of different herbicides (Simazine, Napropamid, Paraquat), fungicides (Captan and Mancozeb) and insecticides (Fenitrothion and Dimethoate) on the number of soil fungi in the soil under apple trees.

MATERIAL AND METHOD

The trial was set up on the alluvium soil type (pH_{nKCl} — 5.8, humus — 0.98%, N-0.04%, P_2O_5 — 14.80 mg 100 g^{-1} soil, K_2O — 16.80 100 g^{-1} soil) of the Experimental Farm of the Fruit and Grape Research Centre in Čačak, in a randomized block design with three replications. The experimental plot size was 20 m^2. Seedlings of the Idared apple variety were used as test plants and treated in early spring with the following pesticides:

Herbicides: Simazine — 4 dm^3 ha^{-1}, Napropamid — 9 dm^3 ha^{-1}, Paraquat — 4 dm^3 ha^{-1}

Fungicides: Captan — 0.2%, Mancozeb — 0.2%;

Insecticides: Fenitrothion — 0.2%, Dimethoate — 0.15%.

Once a month, four times during the growing season, soil sampling was performed for determining the soil fungi number.

The soil fungi number was determined by an indirect method of addition of 0.5 cm^3 10^{-5} dilution on the Czapek agar.

The data obtained were processed by the variance analysis method and the Lsd test was used to perform testing of the significance of differences.

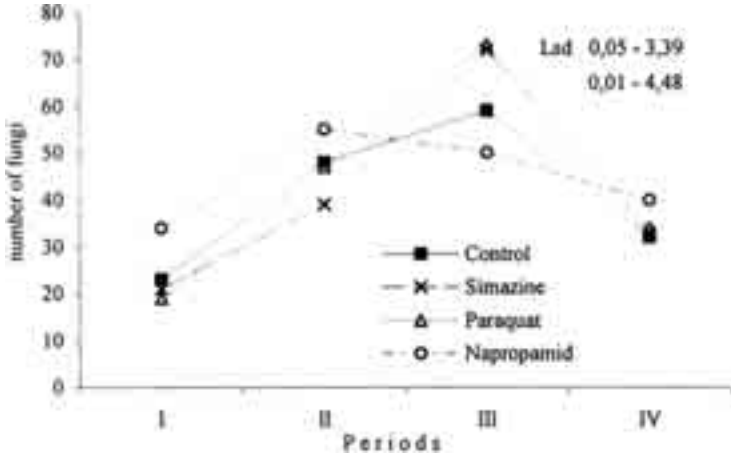
INVESTIGATION RESULTS AND DISCUSSION

Based upon the analysis of variance of the experimental data obtained, we conclude that the effect of the herbicides, fungicides and insecticides used on the number of soil fungi depended not only on their type, but also on the period of sampling for the analysis.

Besides Napropamid, in initial stages of the growing season, the rest of the herbicides used considerably decreased the number of soil fungi, Paraquat

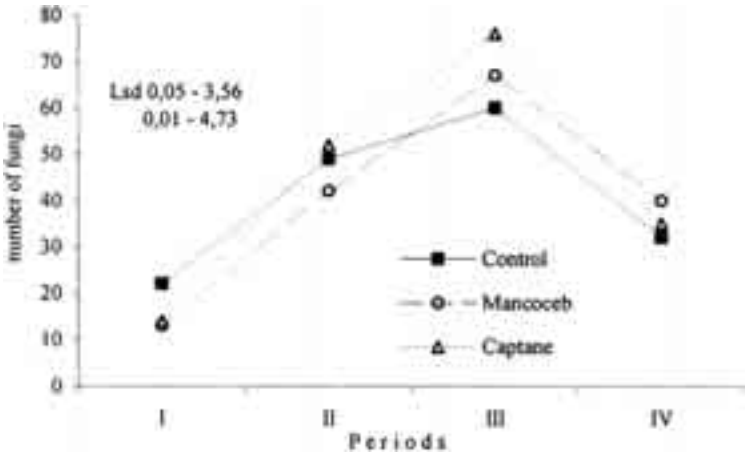
being the leading one in that respect (Graph. 1). After Čulakov et al. (1975), the use of this preparation affects the cell membrane permeability, indirectly impacting a decline in the number of this group of microorganisms. A decline in soil moisture during the second investigation period resulted from a pronounced depressive effect of Simazine as opposed to Napropamid and Paraquat the use of which resulted in increased numbers of this group of microorganisms. The increase in the number of soil fungi in the presence of optimal rates of the preparations was recorded by Chopa and Magu (1985), who associated it with the cometabolic effect in the soil, indirectly affecting the vitality and tolerance of soil fungi to herbicides. At the end of the growing season, excepting Napropamid, a moderate loss of the effects expressed was registered.

In terms of the growing season, the number of soil fungi increased till the third investigation period, whereas the lowest number was recorded at the end of the growing season being in correlation with the plant activity, that is with the amount and value of root exudations as potential food sources for this group of microorganisms (Yemtsev and Đukić, 2000).



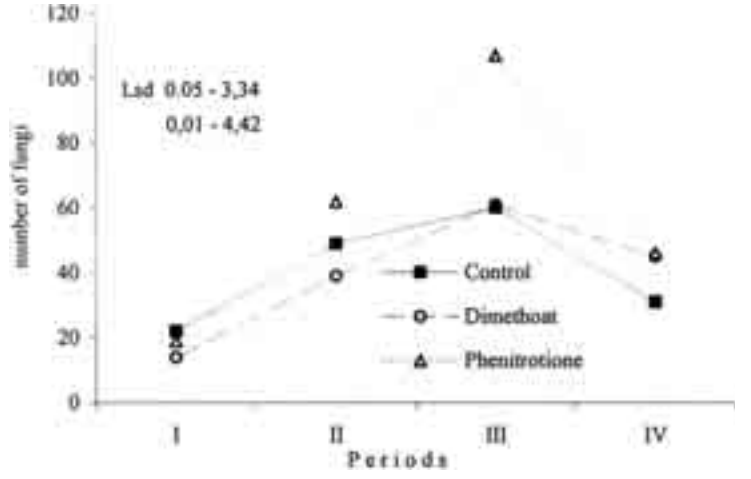
Graph. 1. Effect of herbicides on the number of soil fungi (10^5 g^{-1} soil)

The fungicides used, particularly Mancozeb, in the initial vegetation stages, significantly affected a decline in the number of soil fungi being in accordance with the results obtained by Kling and Jacobsen (1997), who underlined a significant effect of the fungicides on the growth reduction of hyphae and their division, as well as on the decrease in the activity of enzymes responsible for decomposition of these pesticides. During their further determination over the sampling periods, there was a rise in the number of fungi in all variants and a loss of the fungicide effect of the preparations being expressed till the end of the growing season (Graph. 2). Similar impacts of aftereffects of fungicides on the increase in the number of fungi were highlighted by Wainwright and Pugh (1975).



Graph. 2. Effect of fungicides on the number of soil fungi (10^5 g^{-1} soil)

In the first investigation period the Dimethoate insecticide highly significantly decreased the number of fungi, whereas the effect of Fenitrothion was statistically insignificant (Graph. 3). In the second and especially in the third period, a gradual loss of a depressive effect of dimethoate and a pronounced stimulative effect of Fenitrothion were recorded. At the end of the growing season, in spite of the decline in the fungi number, the stimulative effect of the insecticides used was still present. To that end are also the results of other authors who point out that with the extension of the incubation period, the number of soil fungi in the conditions of organophosphorous insecticide application increase, resulting as explained from stimulation of mineralization processes, respiration and oxidoreduction processes in the soil (T u, 1970, J e n k i n s o n, 1976).



Graph. 3. Effect of insecticides on the number of soil fungi (10^5 g^{-1} soil)

CONCLUSION

— The number of soil fungi depends not only on the type of pesticides used, but also on the growing season of the plants cultivated and the time of their determination;

— Of all the herbicides used, the highest and longest depressive effects on the development of soil fungi was registered with Simazine, the effect of Napropamid being the smallest;

— Both fungicides applied perform inhibition of soil fungi development during the first two months following their application;

— The smallest and shortest inhibitory effect on soil fungi was expressed by the insecticides used, Fenitrothion in particular.

A general conclusion could be made, being that the fungi belong to the group of microorganisms that after an initial sensible response to the presence of pesticides in the soil very rapidly establish normal metabolism, indicating that this parameter of soil biologic activity must be taken into account during monitoring of pesticide pollution of soil.

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ЗЕМЉИШНЕ ГЉИВЕ КАО ПОКАЗАТЕЉИ ЗАГАЂЕНОСТИ ЗЕМЉИШТА ПЕСТИЦИДИМА

Лека Мандић[1], Драгутин Ђукић[1] и Снежана Ђорђевић[2]
[1] Пољопривредни факултет, 32000 Чачак, Србија и Црна Гора
[2] Пољопривредни факултет, Београд, 11081 Земун, Немањина 6, Србија и Црна Гора

Резиме

Земљишне гљиве, са израженом ензимском активношћу и високим осмотским потенцијалом, представљају значајан показатељ негативних утицаја различитих пестицида на агро-екосистем као целину. У том погледу, извршен је оглед на алувијалном типу земљишта са циљем да се испита утицај различитих хербицида (Simazine, Napropamid, Paraquat), фунгицида (Captan и Mancozeb) и инсектицида (Fenitrothion и Dimethoate) на једном броју земљишних гљива под стаблима јабука.

Број земљишних гљива одређиван је током четири сезоне гајења индиректном методом додавања разблаживача на Чапек агар.

Резултати проучавања показују да гљиве које припадају групи микроорганизама који, после једног почетног осетљивог одговора на присуство пестицида у земљишту, врло брзо успостављају нормалан метаболизам који им омогућује да чак постају многобројнији. У том погледу, фунгициди и пестициди показали су се прилично успешнима.

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Stevan M. Jasnić[1], *Vera B. Stojšin*[2], *Ferenc F. Bagi*[2]

[1] Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia and Montenegro
[2] Faculty of Agriculture, Trg D. Obradovića 8, 21000 Novi Sad, Serbia and Montenegro

SUGARBEET ROOT ROT IN DROUGHT CONDITIONS*

ABSTRACT: In recent years several types of sugarbeet root rot have occurred in our country causing significant economic damage. The most frequent symptoms are leaf chlorosis and brown-black wet necrosis of the root. The necrosis spread through the entire root and vascular strands.

In the course of this study *F. oxysporum* was the most frequently isolated from infected sugar beet roots. The incidence of other fungi (*Fusarium solani, Rhizoctonia solani* and *Macrophomina phaseolina*) was much lower and it depended on weather conditions. High temperatures occurring during dry years encourage the development of *F. oxysporum*, the causer of sugar beet root rot.

In 2000, an extremely dry year, plant vitality was satisfactory in the experiment with irrigation and the average root rot incidence was low (2,91%). In the nonirrigated variant the average incidence was high (71,02%).

It may be concluded on the basis of the results from five years (2000—2004) that the major causal agents of sugarbeet root rot in our country are species from genus *Fusarium*, especially *F. oxysporum*. *Fusarium* wilt and root rot are due to the increased frequency of dry and warm years.

KEY WORDS: sugarbeet, root rot, *Fusarium oxysporum, Fusarium solani, Rhizoctonia solani*, *Macrophomina phaseolina*, drought conditions

INTRODUCTION

In recent years, several types of sugarbeet root rot have occurred in our country causing significant economic damage (Stojšin et al., 1999; Jasnić et al., 2001; Stojšin, 2003; Marić, Stojšin, 2004). Pathological changes occurring on sugarbeet roots may be caused by parasitic and non-parasitic factors. Phytopathogenic fungi play a primary role in the occur-

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rence of these diseases. According to the researchers who have studied agents of root rot, *Fusarium* rot is the major root disease in our country (Marić et al., 1970; Marić, 1992; Stojšin et al., 2000; Jasnić et al., 2001; Pejović et al., 2003; Stojšin, 2003; Marić, Stojšin, 2004). *Fusarium* rot and charcoal rot (*Macrophomina phaseolina*) cause the most extensive damage (Marić et al., 1970; Stojšin, 1993; Stojšin, 1999). Other fungi such as *Thanatephorus cucumeris* (anamorph *Rhizoctonia solani*), agent of brown rot, *Streptomyces scabies*, agent of root scab, *Pleospora bjoerlingii* (anamorph *Phoma betae*) etc. occur periodically on individual sugarbeet plants and they do not cause great damage (Marić, 1992; Marić, Stojšin, 2004).

The intensity of the occurrence of sugarbeet root rots and composition of mycoflora depend to a large extent on weather conditions. The most extensive damages have occurred under conditions of dry and warm summers, caused most frequently by *Fusarium* root rot (Stojšin, 1993; Jasnić et al., 2001; Stojšin, 2003; Jasnić et al., 2004). In view of the increasing importance of *Fusarium* root rot in our country, in this paper we discuss the disease symptoms, agents and their frequency and the weather conditions that encourage the occurrence of root rot.

MATERIAL AND METHODS

Root sampling

Sugarbeet root rot was registered at several locations across the Vojvodina province and monitored in the 2000—2004 growing seasons. Root samples were taken at different locations in order to identify the causal agent(s) and study the disease symptoms. Roots were sampled at random, and the roots having typical disease symptoms were used for isolation and determination of causal agent(s).

Isolation of fungi

Pieces of tissue 0.5 x 0.5 cm were excised at the border between healthy and infected portions of sugarbeet roots. These pieces were immersed into $HgCl_2$ sublimate for half a minute, for surface sterilization, and then rinsed with sterilized water. The sterilized pieces were placed on PDA medium in sterilized Petri dishes and kept at 25°C in a thermostat for fungal isolation. After the development of fungal colonies, the isolates were sieved to make pure cultures which served for the determination of fungi.

The determination of the fungi from genus Fusarium

Pure cultures of the fungi from genus *Fusarium* were transferred to a carnation medium (CLA) and kept under black light to stimulate fructification.

The determination of the fungi was performed on the basis of the appearance of fungal colonies, conidia and conidiophores, and the development of chlamidospores (Nelson et al., 1983; Butgess et al., 1988, 1991).

The effect of irrigation on the occurrence of root rot

Irrigation is a practice that protects sugarbeet roots from rotting during a dry period. The effectiveness of irrigation in the prevention of root rot was assessed in a sugarbeet irrigation trial conducted at Rimski Šančevi experiment field. The sugarbeet variety Delta was used in the trial. Nine irrigations were performed (12 May, 2 June, 12 June, 22 June, 5 July, 18 July, 26 July, 4 August and 14 August 2000) with a total of 390 mm of water/ha. Individual irrigation rates varied between 30 mm and 50 mm per hectare. Conventional cultivation practices were applied. The previous crop was wheat. The control variant included the same cultivation practices, but without irrigation. It simulated drought conditions since the natural rainfall during the growing season (April—September) was 149 mm, i.e., 210 mm less than the 30-year average.

The harvest was performed on 18 October 2000. The harvested roots from both variants were checked for the signs of infection. After the check, infected roots were randomly sampled for isolation and determination of causal agents of root rot.

RESULTS

Disease symptoms

The observed symptoms of root rot were different and they were placed in three groups:

— Brown-black wet necrosis of the root. The necrosis starts at the tip of the root and it spreads to the crown, i.e. through the entire root. The infected root perishes. A root cross section shows a wet, brown to black necrosis of the tissue, which spreads to vascular strands. Infected plants may be recognized by chlorotic leaves. These leaves wilt gradually, to become dry and necrosed.

— Symptoms on root surface. The infected root becomes grayish, loses turgor and wilts. A longitudinal cross section shows a brown to black necrosis of vascular strands. This type of necrosis is called „dry rot". The infected roots do not perish but remain in the soil as wilted, shriveled and frequently covered with colonies of saprophytic fungi from the genera *Aspergillus* and *Penicillium*. The leaves of the infected plants are chlorotic, but they do not dry and perish.

— Dark brown, irregular-shaped, necrotic spots on root surface. The necrosis typically remains on the surface without extending into the root. The inner part and vascular strands of the root remain uninfected. Infected plants have green leaves and cannot be distinguished from completely healthy ones.

Incidence of fungal species isolated from infected sugarbeet roots

Table 1 shows the incidence of fungal species isolated from infected sugarbeet roots in the period 2000—2004.

Table 1. Incidence of the fungal species isolated from infected sugarbeet roots (2000 — 2004)

Year	No. of infected roots	Isolated fungi (in percentage)			
		Fusarium spp.	*Rhizoctonia solani*	*Macrophomina phaseolina*	*Other saprophytes*
2000	99	68.8	0.0	2.5	6.0
2001	30	16.7	10.0	0.0	6.7
2002	60	26.3	4.7	4.7	48.3
2003	30	61.9	11.8	7.1	23.3
2004	55	30.9	18.2	0.0	14.5
Average ($\overline{X}$)	—	40.9	8.9	2.8	19.8

The five-year results of isolations from infected sugarbeet roots indicated that the genus *Fusarium* was the most frequent and the genus *M. phaseolina* the least frequent.

Table 2 shows the incidence of the species from the genus *Fusarium* in 2000, 2002 and 2003.

Table 2. Incidence of the species from the genus *Fusarium*

Year	Incidence in percentage	
	Fusarium oxysporum	*Fusarium solani*
2000	98.5	1.5
2002	60.0	40.0
2003	100.0	0.0
Average ($\overline{X}$)	86.2	13.8

Table 2 shows that the species *F. oxysporum* predominated in all experimental years. The species *F. solani* was isolated in 2002, but to a much lower extent.

Effect of irrigation of sugarbeet root rot

Table 3 shows the effects of irrigation of sugarbeet root rot in the year 2000.

Table 3. Effect of irrigation on sugarbeet root rot (2000)

Replic.	Treatment	No. of healthy/infected roots	% of infected roots	Fungi (in %) isolated from infected roots	
				Fusarium spp.	*Penicillium* spp.
I	irrigation	34/0	0.0	0.0	0.0
II	irrigation	34/1	2.85	50.0	3.4
III	irrigation	34/2	5.55	36.0	3.4
Average:		34/1	2.91	28.6	2.3

I	dry farming	25/12	32.43	34.6	28.6
II	dry farming	1/34	97.14	24.4	28.0
III	dry farming	5/30	85.71	25.0	28.9
Average:		10/25	71.02	28.0	21.4

The results in the table illustrate the importance of irrigation in mitigating the incidence of root rot — 2.91% infected roots were found in irrigation as opposed to 71.02% found in dry farming. The analysis of the isolated *Fusarium* species showed that only *F. solani* occurred in irrigation, while in dry farming 40% of the isolated species were *F. oxysporum* and 60% *F. solani*.

DISCUSSION

We have described several *Fusarium* diseases that cause the wilt and rot of sugarbeet roots. Despite their increasing importance, these diseases have not received due attention, especially in our country. One of these diseases, which causes leaf chlorosis and root rot, had been named 'sugar beet yellows'. It was described by Stewart (USA) in 1931. The author found that the disease was caused by the fungus *Fusarium conglutinas* var. *betae.* That species was later on renamed into *F. oxysporum* f. sp. *betae* (Snyder, Hansen, 1940). The disease was subsequently described in other countries, Belgium, the Netherlands, Germany and India, in which it caused significant reduction of sugar content in roots (Whitney, Diffus, 1986).

Besides this species which is widely spread and which is typically a major disease, other *Fusarium* species have been described capable of causing pathological changes of sugarbeet roots. The species *F. avenaceum* caused damping-off of sugarbeet seedlings in India (Mukhopadhyay, 1987). The species *F. culmorum, F. sambucinum, F. solani* and *F. coeruleum* (syn. *F. solani* var. *coeruleum*) caused the rot of root core in the former Czechoslovakia (Kockova-Kratochvilova et al., 1958). Fusarium root rot typically caused by *F. oxysporum* f. sp. *betae* was described in our country in 1967 (Marić et al., 1970; Marić, 1974). Since then, the disease occurred regularly each year but it varied in intensity (Balaž, Stojšin, 1997). Besides that disease, charcoal rot caused by *M. phaseolina* may attain significant proportions while *R. solani* is typically a minor disease (Marić, Stojšin, 2004).

In the course of this study, *F. oxysporum* was most frequently isolated from infected sugarbeet roots. The species *F. solani*, *R. solani* and *M. phaseolina* occurred less frequently. Dominance of *F. oxysporum* as agent of root rot was reported by other authors, too (Snyder, Hansen, 1940; Marić, 1974; Whitney, Diffus, 1986; Ruppel, 1991; Jasnić et al., 2001; Marić, Stojšin, 2004). In the period 2000—2004 (Table 1), the incidence of *Fusarium* species ranged from 68.8% in 2000 to 16.7% in 2001. *Fusarium* incidence increased sharply in dry years such as 2000 and 2003, and it decreased (16.7%—30.9%) in years with favorable conditions and evenly distributed rainfall. The incidence of other fungi (*R. solani* and *M. phaseolina*)

was much lower and it depended on weather conditions. Many authors associate the incidence of fungal agents of root rot with prevailing weather conditions (Šenčeno, Signaeskaja, 1962; Toporovskaja, 1969; Marić, 1974; Balaž, Stojšin, 1997; Jasnić et al., 2001; Jasnić et al., 2004). These authors claim that intensive infections occur in dry years, when plant turgor drops considerably. Microorganisms penetrate the root in early summer, particularly in the case of plants weakened by drought. The intensity of fungal penetration corresponds with the intensity and duration of water deficit. Low plant vitality caused by drought or other factors tends to activate the intrinsic mycoflora and trigger the disease as demonstrated by disease symptoms of the whole plant and the root. *Fusarium* species are known as parasites of weak plants. Warm weather, i.e., temperatures over 25°C, encourage the development of *F. oxysporum*, while lower temperatures encourage the development of *F. solani* (Ivanović, Ivanović, Dragica, 2001). High temperatures occurring during dry years favor the development of the former fungal species. *F. solani* incidence increases in rainy years which typically have lower temperatures than normal years. However, even rainy years do not have optimum temperatures for development of this fungus, as confirmed by the 2000 results from our study. In that extremely dry year, when the rainfall during growing season at Rimski Šančevi was 149 mm, or 210 mm below the long-term average, plant vitality was satisfactory in the irrigated variant and the average root rot incidence was 2.91%. In the nonirrigated variant, the average incidence was 71.02%. *Fusarium* species predominated in both variants.

It is difficult to explain the low incidence of *Macrophomina phaseolina* in the years favorable for this fungus such as 2000 and 2003, which had dry and warm summers (Whithey, Diffus, 1986).

Rhizoctonia solani, the agent of brown root rot, occurs in years with warm and humid summers (Parmeter, 1970). Such conditions occur seldom in our country, and this explains the low incidence of *R. solani.* Under the local conditions, 2003 was favorable for this species and its incidence intensified accordingly.

It may be concluded on the basis of the results from several years that the major causal agents of sugarbeet root rot in our country are species from the genus *Fusarium*. Their 5-year average incidence was 40.9%, as compared with 8.9% for *R. solani* and 2.8% for *M. phaseolina*.

In the light of the increasing incidences of *Fusarium* wilt and root rot in our country, which are due to the increased frequency of dry and warm years, it is necessary to study in more detail the etiology of these diseases and measures for their control.

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ТРУЛЕЖ КОРЕНА ШЕЋЕРНЕ РЕПЕ У УСЛОВИМА СУШЕ

Стеван М. Јаснић[1], Вера Б. Стојшин[2], Ференц Ф. Баги[2]
[1] Научни Институт за ратарство и повртарство, Максима Горког 30,
21000 Нови Сад, Србија и Црна Гора
[2] Пољопривредни факултет, Трг Д. Обрадовића 8,
21000 Нови Сад, Србија и Црна Гора

Резиме

Последњих година различити типови трулежи корена шећерне репе се чешће јављају у нашој земљи проузрокујући значајне економске штете Најчешћи знак обољења је хлороза лишћа оболелих биљака у пољу. На корену ових биљака уочавају се тамно мрке некротичне површине, које се спајају захватајући већи део или целу површину корена. Влажна некроза захвата унутрашња ткива корена и оболели корени труле и пропадају. Ређе се јавља само некроза судова корена. Заражени коренови добијају сивкасту боју, губе тургор и вену. То је тзв. сува трулеж корена шећерне репе.

Из оболелих коренова шећерне репе у петогодишњем периоду (2000—2004) изоловали смо најчешће врсту *Fusarium oxysporum* а у знатно мањој мери *F. solani* и гљиве *Rhizoctonia solani* и *Macrophomina phaseolina* поред низа сапрофитних врста (Таб. 1). Посматрањем петогодишњег периода може се видети различит степен заступљености изолованих врста из рода *Fusarium*, који се креће од 68,8% у 2000. до 16,7% у 2001. години. У сушним годинама као што су 2000. и 2003. година заступљеност врста из рода *Fusarium* се знатно повећава, а у годинама са повољним условима и равномерним распоредом падавина заступљеност гљива из овог рода знатно опада (16,7—30,9%). Заступљеност осталих изолованих гљива (*R. solani* и *M. phaseolina*) била је знатно мања и зависила је такође од временских услова током вегетације.

Слабљење виталности биљака, услед суше и неких других фактора, доводи до активирања гљива из рода *Fusarium* и настајања обољења које се испољава увенућем биљака и симптомима на корену. Познато је да су врсте из рода *Fusarium* паразити слабости који се најбоље развијају на ослабљеним биљкама. Ове чињенице потврђују и резултати наводњавања на појаву трулежи корена у 2000. години. У овој екстремно сушној години, у условима наводњавања, где је виталност шећерне репе била задовољавајућа, трулеж корена била је минимална са просечно 2,91% трулих, док је у сувом ратарењу, без наводњавања, код ослабљених биљака просечна трулеж корена била 71,02% (Таб. 2).

На бази вишегодишњих резултата може се закључити да су најзначајнији проузроковачи трулежи корена шећерне репе гљиве из рода *Fusarium* са просечном заступљеношћу од 40,9%, у односу на просечну заступљеност *R. solani* од 8,9% и *M. phaseolina* од 2,8% у петогодишњем временском периоду (Таб. 1). С обзиром на све већи значај фузариозног увенућа и трулежи корена шећерне репе код нас, због све чешћих сушних година, потребно је детаљније проучавање етиологије обољења и мера сузбијања.

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Stevan M. Jasnić[1], *Miloš B. Vidić*[1], *Ferenc F. Bagi*[2], *Vuk B. Đorđević*[1]

[1] Institute of field and Vegetable Crops, 21000 Novi Sad, Serbia and Montenegro
[2] Faculty of Agriculture, 21000 Novi Sad, Serbia and Montenegro

PATHOGENICITY OF *FUSARIUM* SPECIES IN SOYBEAN*

ABSTRACT: The paper describes the symptoms of the *Fusarium* wilt and necrosis of root and lower stem of soybean, which include leaf chlorosis, wilt of the apical portion of the plant, necrosis of the root and lower stem, and wilting of the whole plant. The pods are often poorly developed. The seeds may be smaller and lighter in the weight and infected, as well.

Isolated from diseased soybean plants were the species *Fusarium avenaceum, F. equiseti, F. oxysporum* and *F. poae.* Pathogenicity tests under artificial infection conditions showed *F. oxysporum* (isolate S/1) to be the most pathogenic among of the four investigated species. The other species proved much less pathogenic.

KEY WORDS: soybean, *Fusarium avenaceum, F. equiseti, F. oxysporum, F. poae*, pathogenicity

INTRODUCTION

The *Fusarium* wilt and necrosis of root and lower of soybean (abbreviated to FWNS) is an important disease in many countries. It can cause great damage, as it may reduce the average yield of soybean by up to 59% (Sinclair and Backman, 1989). The fusariosis of soybean was first recorded in 1917 in the U.S. (Cronwell, 1917) and has since been reported in many parts of the world (Sinclair and Backman, 1989). In our country, the disease was first observed and described in 1964 by Aćimović (1988), and later by Tošić et al. (1986) as well. These authors identified *Fusarium* sp. as the causal agent of the disease without specifying which of the species in particular were responsible for causing it.

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In Serbia and Montenegro, the FWNS occurs sporadically and with varying intensity. The severity of attack mainly depends on weather conditions during the growing season. There are no data available on the specific species that cause soybean fusariosis in the country and their importance and pathogenicity to soybean.

Because of this, the objective of our study was to identify the species of the genus *Fusarium* that cause the FWNS and to investigate their pathogenicity.

MATERIALS AND METHODS

The FWNS was observed on soybeans at some locations in the province of Vojvodina. Samples of soybean plants showing symptoms of the disease were collected in order to determine and identify the causal organism responsible. The randomly selected diseased plants were used to isolate the fungi causing the disease.

Isolation of fungi

Using a scalpel, pieces of tissue 0.5 x 0.5 cm in size were cut out of the marginal zone between the healthy and diseased stem tissue. The pieces were then immersed in a sublimate ($HgCl_2$) for about half a minute for external desinfection purposes, after which they were rinsed with sterile water. The sterilized stem pieces were then placed in Petri dishes filled with a potato dextrose agar (PDA) medium and used for isolation of fungi. The Petri dishes were kept in a thermostate at 25°C. After the development of colony the usual phytopathological methods were applied to obtain pure, monosporous culture of isolates and their determination.

Determination of species from genus Fusarium

The monosporous cultures of *Fusarium* fungi were transferred onto a medium consisting of water, agar and carnation leaves, known as the CLA medium (Fisher et al., 1982). The isolates were grown at room temperature under artificial lighting with ultraviolet light added. The source of light were three 40W neon tubes and a black tube emitting the so-called black light (Philips TLD 36W/08). Growing fungi from genus *Fusarium* on the CLA medium in the above manner promotes their sporulation and pigmentation.

Ten to 14 days after the incubation, the isolates of monosporous cultures were used for further study of morphological characteristics, species determination and pathogenicity tests.

Taxonomic characteristics were determined based on the appearance of the colony of fungi on the PDA medium and the formation of conidia, conidiophores and chlamydospores on the CLA medium (Nelson et al., 1983; Burgess et al., 1988; Burgess et al., 1994).

Pathogenicity tests

The pathogenicity of the isolates of fungi was tested in several ways:
— by soybean seed inoculation on filter paper;
— by sowing inoculated seeds in sterile soil;
— by sowing uninfected seeds in artificially infected soil.

The following *Fusarium* isolates were used for pathogenicity tests: S/5, S/8, S/1, S/2 and S/10. They differed from each other in color and colony appearance and were all obtained from diseased stems except the isolate S/2, which was obtained from a wilted soybean seedling.

The conidia suspension for inoculation was prepared by pouring 50 ml of sterile water into each of the Petri dishes containing 14-day-old *Fusarium* isolates, stirring the mixture with a sterile glass stick, and pouring it into a glass. The concentration of conidia in the suspension was determined using Türk-Bürger's plate for spore count. It was set to $1x10^6$ conidia/ml.

Test on filter paper

Seeds of the soybean cultivar Ravnica were sterilized for three minutes with a 1% solution of sodium hypochlorite, rinsed twice with sterile water, and then dipped in the conidia suspension of the each of investigated isolate for a period of five hours. After that, the seeds were placed on wet filter paper in four Petri dishes, each representing one replicate, with 15 seeds per dish. The seeds were germinated in a thermostate at 25°C. Soybean seeds dipped in sterile water for five hours were used as the control. The number of germinated and rotted seeds was determined after seven days, while the number of deformed (diseased) seeds and the total number of healthy seeds were determined after ten days. All the data were statistically processed by the analysis of variance and by determining the significance threshold using Duncan's test.

Planting of infected seeds in sterile soil

For this test, the preparation of conidia suspension and seed inoculation were carried out identically as in the previous one. Inoculated seeds were sown in pots containing sterile soil (10 seeds per pot). The trial included four replicates, so each isolate was used to inoculate 40 seeds in total. The pots were kept in a greenhouse at 22—24°C and watered according to their need. Used as the control treatment were the seeds dipped in sterile water and then planted in sterile soil. The number of emerged plants was recorded ten days after planting.

Test in artificially infected soil

Healthy soybean seeds were planted in pots filled with sterile soil, after which the soil was artificially infected by pouring 50 ml of the conidia suspen-

sion into each pot. Ten seeds per pot were planted. The trial had four replicates, so 40 seeds were inoculated with each isolate overall. The pots were kept in a greenhouse at 22—24°C and watered according to their need. Sterilized soil watered with 50 ml of sterile water per pot was used as the control. Ten days after planting, the number of emerged plants was counted. The number of plants that wiled after emergence was recorded after 14 days.

RESULTS

Disease symptoms

During cool and wet springs, wet rotting and damping off of soybean seedlings was observed in inspected soybean fields. The seedlings often died before emerging, still in the soil. The diseased seedlings necrotize and rot in the soil. The emerged seedlings are stunted in growth. The cotyledons are chlorotic, later become necrotic and decay. The diseased seedlings wilt and dry up. This type of symptoms occurs rarely in Serbia and Montenegro. If the diseased seedlings do not decay, they produce plants that have poor development, have stunted growth and form pods with smaller and curved seeds. The seeds of such plants are often infected.

The symptoms may also appear in older plants during mid-growing season under warm weather conditions. One of the typical signs of the disease is leaf chlorosis. The diseased leaves wilt and dry up. Dropping and wilting of the stem tip is another characteristic symptom. The diseased plants may wilt down and dry up completely. Their roots are necrotic and rotten, and the necrosis will often spread to the lower stem. A cross-section of the stem will reveal necrosis of the vessels. The diseased plants develop fewer pods, which contain smaller seeds.

Most frequently isolated from such plants were fungi of the genus *Fusarium.*

Isolation of fungi

The following species were isolated first from the diseased seedlings and then from the infected stems as well: *F. avenaceum, F. equiseti, F. oxysporum* and *F. poae*.

Determination of Fusarium *species*

On the potato-dextrose medium, isolate S/5 formed colonies light yellow to reddish in color. The color of the colony in the medium was pinkish red or brown. On the monophialides conidiophores in the carnation medium, the isolate developed long and slender macroconidia, whose walls were parallel on a portion of the conidium. Microconidia formed very rarely, while chlamydospores did not form at all. Based on morphological characteristics, the isolate S/5 was determined to belong to the species *F. avenaceum.*

Colonies of the isolate S/8 were initially white but became darker with time and turned light brown in the end. In the medium, the colonies were brown as well. On the CLA medium, extremely curved, sickle-shaped macroconidia developed with their characteristic foot-shaped apical cells. Macroconidia did form on the monophialides, while microconidia did not. Chlamydospores formed in chains or clusters. The isolate S/8 was found to be the species *F. equiseti*.

Isolates S/1 and S/2 formed colonies ranging from white to dark purple in color depending on the isolate. On the carnation medium, macroconidia formed orange-colored sporodochia on the monophialides. Most often, they were short and had three septa and a pointed end. They formed a large number of microconidia, clustered into so-called false heads. The microconidia were unicellular and either elyptical or kidney-shaped. The colonies contained numerous chlamydospores. All these characteristics pointed to the isolates being of the species *F. oxysporum*.

On the potato medium, colonies of the isolate S/10 were white in the beginning but turned purple to brown with age. On the carnation medium, few macroconidia developed. This isolate was definitively identified as being of the species *F. poae* because of its characteristic round or lemon-shaped microconidia with a prominent papilla. The isolate did not form chlamydospores.

Pathogenicity test of Fusarium species

The test results are shown in Table 1. The table shows the average values of the four replicates.

Tab. 1. Pathogenicity of *Fusarium* species on soybean

Species	Filter paper test			Test with sterile soil		Inoculated soil	
	Average no. of germinated seeds	Average no. of rotted seeds	Average no. of malformed seedlings	Average no. of healthy seeds	Average no. of emerged plants	Average no. of emerged plants	Average no. of wilted plants
Fusarium avenaceum S/5	14.25	1.75	0.75	12.50**	4.75**	8.25	0.50
Fusarium equiseti S/8	15.00	0.25	2.50**	12.25**	6.50	8.00	0.25
Fusarium oxysporum S/1	12.50**	3.75**	1.50	10.00**	3.75**	9.25	1.75
Fusarium oxysporum S/2	14.00	1.75	2.50**	10.75**	6.00	8.50	0.25
Fusarium poae S/10	15.00	1.75	3.00**	10.25**	7.25	9.50	0.25
Control	15.00	0.25	0.50	14.25	7.25	8.50	0.00
LSD 0.01	1.76	2.95	1.98	1.91	2.43	2.57	2.30
0.05	1.09	1.75	1.48	1.43	1.80	1.86	3.61

Test on filter paper

According to the results of seed inoculation on filter paper shown in Table 1, all of the isolates of fungi from genus *Fusarium* exhibited a greater or lesser degree of pathogenicity, as they all significantly reduced the average number of healthy seeds (10—12.5) relative to the uninfected control treatment (14.25). *F. oxysporum* isolate S/1 proved to be the most aggressive one, as it had the most significant negative effect on seed germination. The rest of the *Fusarium* fungi had no significant influence on germination (Tab. 1).

Planting of infected seeds in sterile soil

As shown in Table 1, *F. avenaceum* (isolate S/5) and *F. oxysporum* (isolate S/1) were highly pathogenic, since they highly significantly reduced the germination of artificially infected soybean seeds, i.e. the number of plants emerged. *F. equiseti* had less negative influence on germination.

Test in artificially infected soil

In this case, the *Fusarium* isolates had no significant influence on seed germination. All of them, however, caused plant wilting after emergence. The average number of wilted plants ranged between 0.25 and 1.75, depending on the species. S/1 was the isolate with the highest level of pathogenicity in this particular test. However, no statistically significant differences in the average number of wilted plants were found related to the uninfected control.

Our results showed that *F. oxysporum*, isolate S/1 had the highest level of pathogenicity in all of the tests. The pathogenicity of the rest of the species (*F. avenaceum* (S/5), *F. equiseti* (S/8), *F. poae* (S/10) and S/2 *F. oxysporum*) varied.

DISCUSSION

In Serbia and Montenegro, the *Fusarium* wilt and necrosis of root and lower stem of soybean is present in some years to a greater or lesser extent. The FWNS was first observed in our country 1964 (A ć i m o v i ć, 1988) and the causal organism was identified as *Fusarium* sp. Ever since, the disease has failed to receive adequate attention, although it does occur from time to time. The causal agents of FWNS, fungi of the genus *Fusarium*, were not determined, nor was their pathogenicity established. After isolation from diseased soybean plants taken from various locations in the Vojvodina province, the species *F. avenaceum, F. equiseti, F. oxysporum* and *F. poae* were identified.

Around 30 species from genus *Fusarium* have been described worldwide as causal agents of soybean fusariosis (S i n c l a i r and D h i n g r a, 1975). However, not all of them are of equal importance. Some are very widespread

and pathogenic, while others are less virulent and have no major economic importance.

According to the literature, *Fusarium solani* f. sp. *glycines* is among the most pathogenic species in North and South America (Nelson et al., 1997; Roy, 1997; Homma et al., 2002). This parasitic species causes a rapid death of soybean plants (Sudden Death Syndrome) and it is responsible for major economic damages. In 2002, for example, it caused an estimated damage of around 157 million USD in some states in the U.S. (Wrather et al., 2003). In the present study, this species was not isolated from the diseased soybean plants, although it has been reported in some European countries (Patkowska, 2001).

In our study, the highest level of pathogenicity in all the tests was exhibited by *F. oxysporum*. This species has been reported as a major pathogen in many other countries of the world (Yasem de Romero et al., 2002; Patkowska, 2001; Reynolds and Potter, 2001; Tenuta, 2004). It was interesting that there exists signicant difference beetwen two *F. oxysporum* isolates (S/1 and S/2). The isolate S/1 obtained from diseased stem was much more virulent than the isolate S/2 from wilted seedlings. The rest of the species we isolated exhibited a considerably lower degree of pathogenicity and probably have no major importance in the etiology of the disease. Some of them have also been isolated in other countries but have not exhibited a significant level of pathogenicity, which supports the findings of the present study (Warren and Kommendahl, 1971; Vardaniya, 1971).

Other species mentioned in the literature as important soybean pathogens are *F. semitectum, F pallidoroseum, F. tucumaniae* and *F. virgiliforme* in South America and India (Goulart, 2000; Gupta and Aneja, 2001; Skandiani et al., 2004; Aoki et al., 2004). None of them have been isolated in Serbia and Montenegro, as their development requires warm and humid weather.

Since dry and warm summers are becoming more and more common in our country, special attention has to be paid to the species *Fusarium oxysporum*, which exhibited a significant amount of pathogenicity to artificially infected soybeans. This species is known as a weakness parasite that attacks plants weakened by unfavorable environmental conditions.

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ПАТОГЕНОСТ *FUSARIUM* ВРСТА НА СОЈИ

Стеван М. Јаснић[1], Милош Б. Видић[1], Баги Ф. Ференц[2], Вук Б. Ђорђевић[1]
[1] Институт за ратарство и повртарство, 21000 Нови Сад, Србија и Црна Гора
[2] Пољопривредни факултет, 21000 Нови Сад, Србија и Црна Гора

Резиме

Фузариозна увелост, некроза корена и приземног дела стабла соје појединих година се јавља и у нашој земљи у већој или мањој мери. Ово обољење се интензивније јавља у годинама са топлим и сувим летима, погодним за развој увелости соје, проузроковане врстама из рода *Fusarium*. Из узорака оболелих биљака са симптомима обољења су изоловане и детерминисане врсте *Fusarium avenaceum, F. equiseti, F. oxysporum* и *F. poae*. У огледима са вештачком инокулацијом соје највећу патогеност испољавао је изолат S/1 *F. oxysporum*. *F. oxysporum* (S/1) је значајно смањио клијавост и ницање биљака соје, а повећао број трулих зрна. Остале врсте испољиле су знатно слабију патогеност.

Проблему фузариозне увелости, некрозе корена и приземног дела стабла соје требало би посветити већу пажњу због могућности увећања значаја овог обољења, услед све чешће појаве топлих и сувих лета, повољних за развој болести.

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Jasmina M. Glamočlija[1], *Marina D. Soković*[1], *Jelena B. Vukojević*[2], *Ivanka M. Milenković*[2], *Dejan D. Brkić*[3], *L. J. L. D. Van Griensven*[4]

[1] Institute for Biological Research „Siniša Stanković", Despota Stefana142, 11000 Belgrade, Serbia and Montenegro
[2] Institute of Botany, Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia and Montenegro
[3] Catholic University of Louvain, Department of Analytical Chemistry, Drug Analysis and Pharmacognosy, Avenue E. Mounier 72, 1200 Brussels, Belgium
[4] Plant Research International, Wagenningen University, The Netherlands

ANTIFUNGAL ACTIVITY OF ESSENITAL OIL *HYSSOPUS OFFICINALIS* L. AGAINST MYCOPATHOGEN *MYCOGONE PERNICIOSA* (MANG)*

ABSTRACT: The most commonly cultivated mushroom species is the *Agaricus bisporus* Lange (Imb). One of the major pathogenic diseases of the cultivated mushroom in Serbia is *Mycogone perniciosa* (Mang). Biological control systems are not much used in mushroom cultivation. Medical and aromatic plants have been placed in the focus of intense studies.

Pure culture of the *M. perniciosa* was isolated from infected *A. bisporus*. The essential oil of *Hyssopus officinalis* L. is used as a potential antifungal agent. The most abundant components in oil are isopinocamphone (43.29%), pinocamphone (16.79%) and b-pinene (16.31%). Antifungal activity of Hyssop was investigated by the modified microatmosphere method. The minimal inhibitory quantity was 5 μL/mL and a minimal fungicidal quantity was 15—20 μL/mL.

There is no report on the use of Hyssop essential oil in mushroom disease.

KEY WORDS: *Agaricus bisporus*, *Hyssopus officinalis*, essential oil composition, antifungal activity, *Mycogone perniciosa*

INTRODUCTION

Agaricus bisporus Lange (Imbach) is a common edible mushroom with major economic value and a cosmopolitan distribution (Kerrigan, 1995). World wide cultivation of the button mushroom is 1.9 million tones in 1998/

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1999 (Van Griensven, 2003). The cultivated mushrooms are subject to various diseases and pests that have the capacity to cause serious crop losses. Many microorganisms, such as fungi, bacteria and viruses attack mushrooms. Fungi are effectively the most important group of pathogens (Fletcher et al., 1986). One of the major pathogenic diseases of the cultivated mushroom is *Mycogone perniciosa* (Mang), commonly known as Wet Bubble Disease (WBD) which caused considerable crop loss (Sisto et al., 1997, Sharma and Kumar, 2000, Bora and Özaktan, 2000, Nanagulyan and Yesayan, 2002).

The symptom of WBD is the development of cauliflower-like distortion on fruit bodies of *A. bisporus* like sclerodermoid masses that are white and fluffy at the beginning, but become brown with age and decay. In the conditions of a very high humidity brown drops develop on the surface of tumour-like bodies. Spores are infectious and spread by water splash and by insect vectors. The primary source of the pathogen is contaminated casing, but the secondary one is caused by facilities, personnel and insect vectors.

Mushroom cultivation in Serbia is still less developed than in other European countries. *M. perniciosa* has a significant influence on the quality and yield of mushrooms.

Very limited numbers of fungicides are available and approved for use in mushroom cultivation. Also, the development of pathogen resistance to the fungicides was closely related to the frequency of their use (Grogan and Gaze, 1998). Biological control systems, which have been successfully applied to some crops, are not much used in mushroom cultivation. It is difficult to find some safe disease spray to use on mushrooms when they are close to harvest. One possibility might be using herbal spray. Medical and aromatic plants have been placed in the focus of intense studies.

Hyssopus officinalis, L. belongs to the Labiate family of plants, of which numerous species have antiseptic properties against bacteria, fungi and viruses. Hyssop is a perennial sub shrub native to southern Europe, the Mediterranean region, and temperate Asia and naturalized in the United States. The therapeutic activity of the herb of *H. officinalis* has usually been attributed to the components of its essential oil. As a medicinal plant, Hyssop has been used as a carminative, diaphoretic, emmenagogue, expectorant, stimulant, stomacher, and tonic. Leaves have been used as a remedy for asthma, rheumatism, sore throats, wounds, ulcers, and tumours (Lawless, 2002).

In this study, we examined the action of essential oil of *Hyssopus officinalis* against the mycopathogen *Mycogone perniciosa*. The use of natural antifungal compounds in the control of human, animal and plant diseases of microbial origin was reported before (Soković, 2001).

MATERIALS AND METHODS

Essential oil and analysis — Essential oil of *Hyssopus officinalis* L. is commercial sample obtained by the Institute for Medicinal Plant Research „dr Josif Pančić", Belgrade.

Essential oil was investigated for its composition by the use of analytical GC/FID and GC/MS technique. For these purpose HP 5890 series II gas chromatograph, equipped with split-split less injector, fused silica capillary column (25 m x 0.32 mm), coated with cross-linked methyl silicone gum (0.5 μm film thickness), and FID was employed. Essential oil solutions in ethanol (1%) were injected in split mode (1:30). Injector was heated at 250°C, FID at 300°C, while column temperature was linearly programmed from 40—280°C (4°/min). GC/MS analyses were carried out on a HP-GCD, equipped with split-split less injector, fused silica capillary column (50 m x 0.2 mm) PONA, coated with cross-linked methyl silicone gum (0.5 μm film thickness). The chromatographic conditions were as above. Transfer line (MSD) was heated at 280°C. EIMS spectra (70eV) were acquired in scan mode in m/e range 40—300.

The identification of individual constituents was made by the comparison of their retention times with those of analytical standards, and by computer searching, matching mass spectral data with those held in Wiley/NBS library of mass spectra. For quantification purposes area percent reports obtained by FID were used (Adams, 1995).

Fungal strain and media — Samples of diseased mushrooms were collected from mushroom farms in Serbia. Pure culture of the *M. perniciosa* was isolated from diseased *A. bisporus* in the Mycological Laboratory, Institute for Biological Research „Siniša Stanković". The mycopathogen was maintained on potato dextrose agar (PDA). The cultures were stored at 4°C and subcultured once a month (Booth, 1971).

Test for antifungal activity — The modified microatmosphere method (Zollo et al. 1998) was used for the investigation of antifungal activity of essential oils. Petri plates measuring 50 mm were filled with 10 mL potato dextrose agar (PDA) medium and then seeded with a small amount of 7-days--old mycelium culture of the tested fungi. The Petri dishes were then inverted and the determined amount (5—20 μL/mL) of pure oils impregnated on sterile filter paper discs (6 mm in diameter) deposited on the inverted lid. Minimal inhibitory quantities (MIQ) and minimal fungicidal quantities (MFQ) of essential oils were noted every 7 days. MIQ and MFQ are reported as the mean ± SD of three replicates for each concentration (quantities) of oils. The inverted Petri dishes were incubated at 25°C for 21 days.

RESULTS AND DISCUSSION

The results of chemical analysis of essential oil from Hyssop are presented in Table 1. The results suggested that the activity of *H. officinalis* can be attributed to ketons which are the main constituents: isopinocamphone (43.29%), pinocamphone (16.79%) and b-pinene (16.31%).

The essential oil of *H. officinalis* showed a very strong antifungal activity. The minimal inhibitory quantity was 5 μL/mL and minimal fungicidal quantity was 15—20 μL/mL.

Furthermore, antimicrobial activities of *Hyssopus* species were shown in other previous studies (Mazzanti et al., 1998, Renzini et al., 1999). The essential oil of Hyssop had fungistatic action on *Aspergillus fumigatus*. Binding of this oil or some of its constituents to membranes has been found to affect ion exchanges (Ghfir et al., 1994.) The antifungal and fungicidal effects of Hyssop oil and its individual components were studied against plant pathogenic fungi (Letessier et al., 2001). The very strong antifungal potential of Hyssop essential oil can be explained by high amount of ketons which are the main constituents (Griffin et al., 2000).

In the control of WBD Prochloraz manganese, Benzilmidazole fungicides and 1% formalin in treating casing give a good control, but *Mycogone* remains a constant threat. The high toxicity of these fungicides and emerging tolerance of mycoparasites to some fungicides makes it necessary to continue the search for new antifungal substances. During the past years numerous antifungal agents have been formulated and evaluated for use in the management of fungal diseases microwave treatments or some effective antagonistic bacteria (Bora and Özaktan, 2000). Essential oils used in this work could be a very good alternative in treatment of fungal diseases because of their very good antifungal activities.

Table 1. The composition of essential oil of *Hyssopus officinalis.*

Components	*H. officinalis* %	RI
α-thujene	0.25	307
α-pinene	0.95	319
camphene	0.24	340
β-pinene	16.31	386
myrcene	0.54	408
p-cymene	1.07	471
limonene	1.56	481
1,8-cineole	0.49	485
trans-ocimene	0.54	519
γ-terpinene	0.93	545
terpinolene	0.26	608
α-thujone	0.13	642
β-thujone	0.13	667
pinocamphone	16.79	775
isopinocamphone	43.29	809
myrtenal	1.37	864
methyl chavicol	0.07	869
β-bourbonene	1.20	1355
β-elemene	0.09	1375
α-gurjunene	0.18	1421
β-caryophyllene	1.00	1442
allo-aromadendrene	0.63	1546
germacrene D	0.93	1594
bicyclogermacrene	0.12	1632
RI-DB 5		

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АНТИФУНГАЛНА АКТИВНОСТ ЕТАРСКОГ УЉА *HYSSOPUS OFFICINALIS* L. НА МИКОПАТОГЕН *MYCOGONE PERNICIOSA* (MANG)

Јасмина М. Гламочлија[1], Марина Д. Соковић[1], Јелена Б. Вукојевић[2], Иванка М. Миленковић[2], Дејан Д. Бркић[3], Л. Ј. Л. Д. Ван Гриенсвен[4]
[1] Институт за биолошка истраживања „Синиша Станковић”, Деспота Стефана 142, 11000 Београд, Србија и Црна Гора
[2] Институт за ботанику, Биолошки факултет, Универзитет у Београду, Таковска 43, 11000 Београд, Србија и Црна Гора
[3] Catholic University of Louvain, Department of Analytical Chemistry, drug Analysis and Pharmacognosy, Avenue E. Mounier 72, 1200 Брисел, Белгија
[4] Plant Research International, Wagenningen University, Холандија

Резиме

Agaricus bisporus Lange (Imb) је најчешће комерцијално гајена јестива гљива. Различити микроорганизми гљиве, бактерије и вируси су изазивачи болести у гајилиштима шампињона. *Mycogone perniciosa* (Mang) је изазивач болести познате под називом влажни мехур и најчешћи узрочник губитака у гајилиштима у Србији. Биолошка контрола, која је успешно примењивана на неким пољопривредним културама, није коришћена приликом узгоја гљива. Једна од могућности је примена биљних спрејова. Лековите и ароматичне врсте биљака се интензивно истражују као могући антифунгални агенси.

Узорци оболелих шампињона су сакупљани у гајилиштима у Србији. Културе *M. perniciosa* су изоловане са оболелих плодоносних тела *A. bisporus*. Коришћено је етарско уље *Hyssopus officinalis*. Најзаступљеније компоненте уља су изопинокамфон (43.29%), транс-пинокамфон (16.79%) и б-пинен (16.31%). Антифунгална активност етарског уља изопа испитивана је модификованом „микроатмосфера”-методом. Минимална инхибиторна количина је била 5 μL/mL, а минимална фунгицидна количина 15—20 μL/mL.

Велик број препарата је направљен и примењен за контролисање обољења печурака: фунгициди, примена микроталаса или дејство неких антагонистичких бактерија. Досад није било саопштења о примени етарског уља изопа против изазивача болести гајених гљива.

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Ida J. Leskošek-Čukalović, Viktor A. Nedović

Institute of Food Technology and Biochemistry, Faculty of Agriculture,
University of Belgrade, Nemanjina 6, PO Box 127,
11081 Belgrade—Zemun, Serbia and Montenegro

IMMOBILIZED CELL TECHNOLOGY IN BEER BREWING — CURRENT EXPERIENCE AND RESULTS*

ABSTRACT: Immobilized cell technology (ICT) has been attracting continual attention in the brewing industry over the past 30 years. Some of the reasons are: faster fermentation rates and increased volumetric productivity, compared to those of traditional beer production based on freely suspended cells, as well as the possibility of continuous operation. Nowadays, ICT technology is well established in secondary fermentation and alcohol-free and low-alcohol beer production. In main fermentation, the situation is more complex and this process is still under scrutiny on both the lab and pilot levels.

The paper outlines the most important ICT processes developed for beer brewing and provides an overview of carrier materials, bioreactor design and examples of their industrial applications, as well as some recent results obtained by our research group. We investigated the possibile applications of polyvinyl alcohol in the form of LentiKats®, as a potential porous matrices carrier for beer fermentation. Given are the results of growth studies of immobilized brewer's yeast *Saccharomyces uvarum* and the kinetic parameters obtained by using alginate microbeads with immobilized yeast cells and suspension of yeast cells as controls. The results indicate that the immobilization procedure in LentiKat® carriers has a negligible effect on cell viability and growth. The apparent specific growth rate of cells released in medium was comparable to that of freely suspended cells, implying preserved cell vitality. A series of batch fermentations performed in shaken flasks and an air-lift bioreactor indicated that the immobilized cells retained high fermentation activity. The full attenuation in green beer was reached after 48 hours in shaken flasks and less than 24 hours of fermentation in gas-lift bioreactors.

KEY WORDS: beer, immobilized yeast, gas-lift bioreactor, alginate beads, PVA LentiKat® carriers

INTRODUCTION

Brewing is one of the oldest biotechnologies with the history dating back more than 8000 years. For centuries, people were brewing beer empirically, using the old recipes. Then, in the 18th century, the biologists began to study

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the process of brewing and to discover some of its basic principles. These early discoveries led to a better understanding of the process and, ultimately, to the development of the brewing industry as we know it today. Since the Second World War, the brewing industry, like all other branches of the processing industry, began to utilize larger production units and to reduce production costs.

Fermentation is the essential part of the brewing process. The main fermentation is responsible for the formation of most flavor compounds, while the secondary fermentation provides beer maturation and final beer sensory properties. These are, at the same time, the most time consuming steps in the overall beer production. From the very beginning of the modern brewing age, science and technology have had an important influence on the development of the novel technical solutions and the improvement of the existing ones. They led to the design of new accelerated fermentation methods (which incorporate improved batch bioreactors, ranging from open, relatively shallow tanks to large cylindro-conical fomenters) and to the development of continuous beer fermentation processes.

Since the beginning of the 20th century, many different systems that use suspended yeast cells have been developed. First fully continuous process for beer fermentation (van Rijn), with six vessels in series, was patented in 1906. During the 1950s and 1960s, several of them have been in commercial use. They had clear advantages over the traditional batch techniques: lower investment costs, lower extract losses, lower fuel and power consumption and, finally, billing practice, etc. (B i s h o p, 1970; T h o r n e, 1968). However, they were not commercially successful due to many practical disadvantages. Depending on the system in use, they caused numerous problems. The list of disadvantages was very long: inflexibility in the output rate or in the ability to change beer type, flavor-matching, need for a high standard of hygiene, possibility of yeast mutation, need for an extra procedure, need for an extremely flocculent yeast, wort storage requirements, cost of technical support, etc. (T h o r n e, 1968). Therefore, by the end of the 1970s most of the operating systems were closed down. The famous exception is the Coutts' process in Dominion Breweries, New Zealand (H o u g h, 1982). It was obvious that the right solution had to be found. Immobilized processes were expected to be a viable one.

APPLICATION OF IMMOBILIZED CELL TECHNOLOGY IN BREWING INDUSTRY

Traditional beer fermentation technology uses freely suspended yeast cells to ferment wort in a non-stirred batch reactor. The primary fermentation for lager beer takes approximately 5 to 7 days with a subsequent secondary fermentation (maturation) of several weeks. Modern batch fermentation technology can reduce the production time (main and secondary fermenation) of lager beer to 10—12 days, but this is the best it can do. Immobilized cell technology (ICT) is able to produce lager beer in less than 2 days.

ICT processes have been designed for different stages in the beer production/fermentation process. The most important of these are: bioflavouring during the maturation, main fermentation and fermentations for the production of alcohol-free or low-alcohol beers. The main objective of flavour maturation is the removal of the vicinal diketones diacetyl and 2,3-pentanedione, and their precursors a-acetolactate and a-acetohydroxybutyrate. Diacetyl is reduced by yeast reductases to 2,3-butanediol via acetoin and 2,3-pentanedione to 2,3-pentanediol via acethylethylcarbinol. The conversion of the α-acetohydroxy acids to the vicinal diketones in traditional maturation process is the rate limiting step. It is characterized by a near-zero temperature, low pH and low yeast concentration, resulting in a maturation period of 3 to 4 weeks. Using immobilized yeast cells this period can be reduced to 2 hours. The reaction step is accelerated by heating the beer after yeast separation to 80—90°C during a period of a few minutes (P a j u n e n, 1995; B a c k et al., 1998).

The traditional technology to produce alcohol-free or low-alcohol beer is based on the suppression of alcohol formation by restricted batch fermentation or on the removal of ethanol, using membrane, distillation or vacuum evaporation processes (M u l l e r, 1990; N a r z i s s et al., 1992). In both cases, the problem is either the undesirable wort aroma from wort aldehydes, which are reduced only to a limited degree, or the insufficient process selectivity. A short-contact with the immobilized yeast cells at a low temperature solves these problems (M e n s o u r et al., 1997; N a v r á t i l et al., 2000).

The design and optimization of an ICT process for the combined main and secondary fermentation remains a challenging task. In spite of much experimental work, ICT processes have not yet been adopted in the brewing industry. When compared to the secondary fermentation, the main fermentation is significantly more complex and has various side reactions that are important for final beer quality. It was reported that insufficient free amino nitrogen consumption by immobilized yeast cells, coupled with mass transfer restrictions and reduced cell growth in immobilized conditions, causes an unbalanced flavor profile of the final beer. Immobilized systems, based on packed bed fermenters with solid carriers for yeast cells and suitable for secondary beer fermentation, were shown to be inappropriate. The reasons include flavor problems, yeast viability and carrier price.

PROCESS DEVELOPED FOR BEER FERMENTATION

The brewing industry has been showing interest in ICT since its appearance and particulary after the introduction of alginate as a carrier (W h i t e and P o r t o, 1978). One of the first processes for rapid lager fermentation was developed by the research team at „Kirin Brewery Company", Japan. It was a multistage ICT system for fermentation and maturation. The process consisted of three bioreactors. The first was an aerated continuously stirred tank for yeast growth. It was followed by two packed bed reactors, in series, for main fermentation and heat treatment for a-acetolactate conversion into diacetyl. Finally, there was a packed bed reactor with immobilized yeast for maturation.

Using this process, beer could be produced within three to five days. The first immobilization method that was used was entrapment in alginate beads, but because of the problems it caused (decreased fermenting capacity, insufficient mechanical strength, swelling of the carrier leading to plugging of the bioreactor, etc.) it was replaced by ceramic beads developed by Kirin („Bioceramic®") (Yamauchi, 1994). The Company set up a small commercial unit on the Saipan island, producing 1850 hl per year. However, their brewing proved to be short lived. The lager beer they produced was sensorly acceptable, but somewhat different from the conventional batch beer. In addition, they could not reach one fifth of the designed output capacity without experiencing deterioration in yeast fermentation activity. Finally, the energy costs and the beer losses were high with centrifuged yeast (Inoue, 1995).

A research group at „Labatt Breweries" (Interbrew, Canada) in collaboration with the Dept. of Chemical and Biochemical Engineering at the University of Western Ontario went in another direction. They applied k-carrageenan beads as a carrier in a draft tube bed reactor. The advantage of k-carrageenan as the carrier material is its density, which is close to that of water and thus minimizes the energy required for fluidization. Small bead size (0.2 to 1.4 mm), fluidized bed design (feed gas mixture of 2—5% of air and CO_2) and a better mass transfer were expected to solve the problems with insufficient amino acid consumption and unbalanced flavor profile. Pilot scale research showed that in continuous fermentation, full attenuation was reached in 20—24 hours and the flavor profile of the beer was acceptable and similar to the batch fermented beer (Mensour et al., 1995; Pilkington et al., 1999).

„Meura Delta", Belgium, proposed a completely different concept. Their goal was to solve the problem of unsuitability of alginate beads in a packed bed reactor and carrageenan beads in a fluidised bed reactor and so they developed a tubular matrix of sintered silicon carbide installed into a loop bioreactor. The system has been used for maturation, alcohol-free beer production and for main fermentation (Van De Winkel, 1995; Andries et al., 2000). For the main fermentation of lager beer, two similar bioreactors were used in series. The first bioreactor was operated at an apparent attenuation level of 40%, and the complete attenuation was reached in the second bioreactor. The residence time was 22 h per bioreactor, while productivity for one matrix depended of the wort's original gravity: at 12—16° Plato, it was 6.6—9.1 hl per year, respectively. This means that for achieving an annual output of 1 million hl, more than 100000 matrices are needed (Virkajärvi, 2001).

VTT Research Institute (Finland) offered a solution for beer maturation that uses a DEAE-cellulose carrier in a fixed-bed reactor. These results led to industrial applications at the Sinebrychoff's Helsinki brewery in 1990 and later on at the Sinebrychoff's Kerava brewery, where the production levels of 1 million hl per year were achieved (Pajunen, 1995). Using a traditional main fermentation and heat treatment of green beer, the maturation period for diacetyl conversion has been reduced from 3—4 weeks to 2 hours. Later on, this carrier was replaced by cheaper aspen wood chips for yeast cell immobilization (Virkajärvi, 2002). Few years after that, Synebrychoff Brewery (Finland), in collaboration with Guinness, GEA Liquid Processing Scandinavia

and Cultor Corporation of Finland, developed a new ICT process for continuous main fermentation that uses a fixed-bed reactor and DEAE-cellulose at the beginning, and wood chips later on, as carrier materials. Good quality beer and constant flavor profile were achieved at a production time of 20 to 30 hours (Andersen, 1999).

„Alfa Laval and Schott Engineering" developed a maturation system based on porous glass beads. This system has been implemented in several breweries in Finland, Belgium and Germany. The produced beers had overall good analytical and sensorial properties (Dillenhofer and Ronn, 1996).

Yeast immobilized on DEAE-cellulose packed in a column reactor has been successfully applied for controlled ethanol production of low-alcohol and alcohol-free beers (Van Dieren, 1995). This technology has been implemented by Bavaria Brewery (The Netherlands and several other companies, including Faxe (Denmark), Ottakringer (Austria) and a Spanish brewery (Mensour et al., 1997).

Our group started the experiments on application of ICT in beer fermentation in early 90s. The aim was to find the optimal solution for reactor design, carrier selection and immobilization techniques. Practically at the same time when Labatt research group started with their fluidized-bed fermentor with carragenan, our group was using alginate beads in the similar type of a fermentor: a three-phase gas-lift fermentor (Figure 1) (Nedović et al., 1993). A gas-lift reactor retains the advantages of fluidized-beds, such as high loading of solids and good mass transfer properties, and is particularly suitable for applications with low-density carriers. Other important characteristics of gas-lift fermenters are their simple construction, low risk of contamination, easy adjustment and control of the operational parameters, and simple capacity enlargement (Nedović et al., 2002). We set out to systematically investigate the conditions which might influence fermentation kinetics, yeast metabolism and, lastly, the sensory profile of final beer. We focused on porous matrices carriers: medium-viscosity Na-alginate and polyvinyl alcohol in the form of LentiKats®.

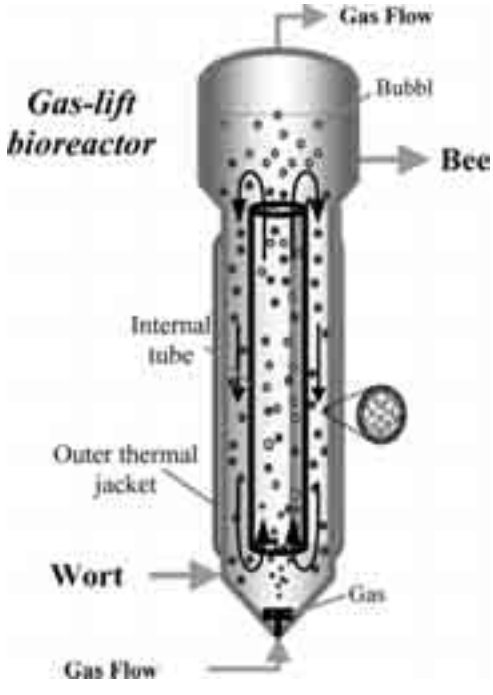

Figure 1. Gas-lift bioreactor system

Polyvinyl alcohol in the form of LentiKats® was reported as one of the promising materials for cell immobilization (Jakel et al., 1998; Jahnz et al., 2001). LentiKats® stands for lens-shaped gel particles, which are produced by new simple gelification technique at room tem-

perature. LentiKats® were investigated as cell carriers in several biological processes, such as bioconversion of glycerol to 1,3-propanediol (Wittlich et al., 1999), cider fermentation (Durieux et al., 1999, 2000, 2002), waste water treatment (Sievers et al., 2002), and production of L-Tryptophane (Klaben et al., 2002). In the present work, we have studied the application of LentiKats® as the potential cell carriers for beer industry.

MATERIALS AND THE METHOD

2.1. *Preparation of microbeads*

Production of alginate microbeads: The technique we used to produce small enought alginate beads (< 1 mm) is the electrostatic droplet generation method. It consists of applying an electrostatic potential between the droplet formation device and the collecting solution, and inducing a charge at the surface of the polymer solution, which results in a decrease in surface tension. Using this method, a significantly greater reduction of droplet size is realized as compared to the one that is achieved using the simple dropping method (Figure 2) (Nedović et al., 2001). Process parameters were: positively charged needle set-up, applied potential: 8 kV, needle size: 27-gauge, electrode distance: 2.5 cm. Polymer/cell suspension was formed by mixing the Na-alginate solution (2% Na-alginate) with the thick brewer's yeast (*Saccharomyces uva-*

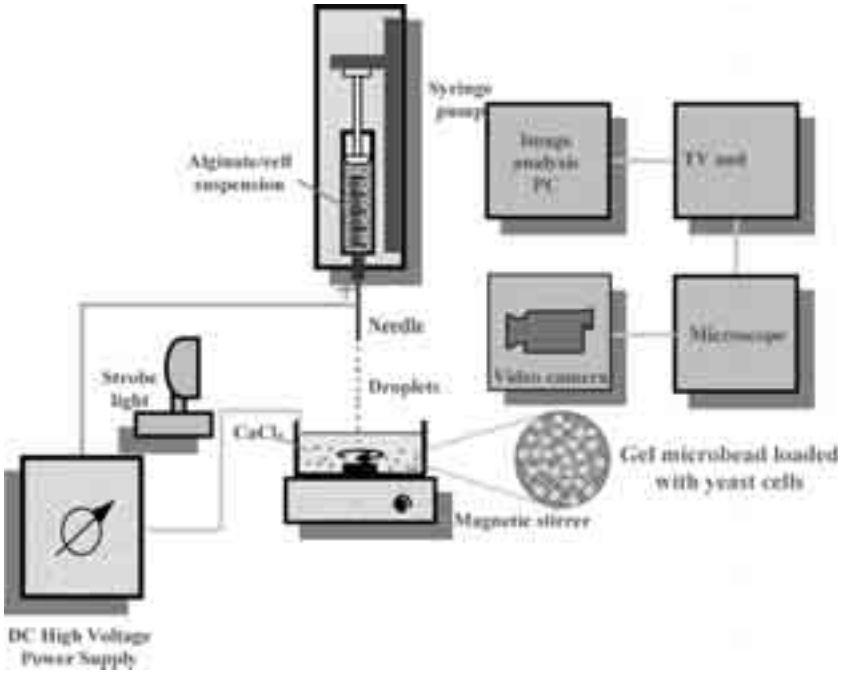

Figure 2. Production of alginate microbeads by electrostatic droplet generation

rum). The resulting microbeads were 0.3, 0.5 and 0.6 mm in diameter and contained immobilized yeast cells at a starting concentration of $2x10^7$ cells/ml.

The PVA LentiKats® were produced by a new, simple gelification technique at room temperature. With LentiKat® Printer, the PVA/yeast cell solution was forced out of the tip of a blunt edge needle (1 mm in diameter) by a syringe, in the form of droplets on Petri dishes. Gelification of the droplets occurred in approximately half an hour at a 75% decrease of the initial mass due to water evaporation. The resulting LentiKat® lenses were about 3.5 mm in diameter and 0.3 mm thick with immobilized yeast cells at a starting concentration of $1x10^7$ cells/ml (Figure 3).

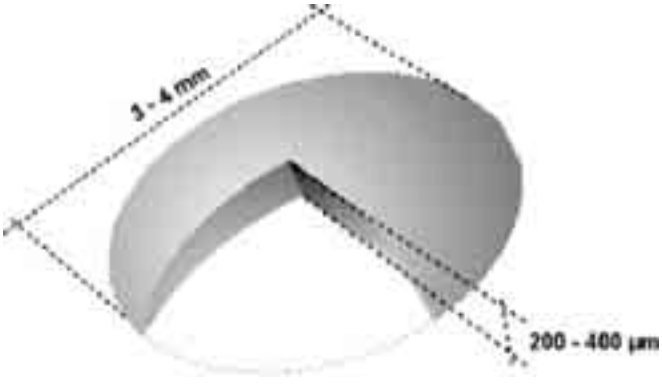

Figure 3. Schematic presentation of a LentiKat® lens

2.2. *Growth studies*

The kinetic parameters of immobilized yeast growth were investigated in a short-term study, by cultivating LentiKat® lenses for 85 hours in shaken flasks. Alginate microbeads with immobilized yeast cells, as well as the suspension of yeast cells at the same starting concentration ($0.5x10^6$ cells/ml) served as controls. LentiKat® lenses, alginate microbeads and the medium were sampled at timed intervals and analyzed for cell viability, concentration and colony distributions.

2.3. *Fermentation studies*

A series of batch fermentations were performed in shaken flasks and in an air-lift bioreactor. The goal was to determine the fermentation activity of brewer's yeast cells, immobilized in LentiKat® carriers, the time necessary to achieve full beer attenuation and to test the stability of LentiKat® carriers in multiple fermentations. In the first set of experiments, fermentation was managed in 500 ml flasks with 210 ml of sterile plant wort (12% extract) and 70 g of LentiKat® lenses. The experiments were performed in duplicates on an orbi-

tal shaker at 115 rpm and 17°C. The concentration of immobilized cells was about $5x10^8$ cells/ml LentiKats®. LentiKat® lenses and the medium were sampled at timed intervals and analyzed. In the second set of experiments, fermentation was managed in internal-loop gas-lift bioreactor with working volumes of 1 dm^3. (Figure 1). Nitrogen was introduced through a glass sparger at the bottom of the reactors, at the gas flow rate of 240 ml/min. The initial concentration of immobilized cells was about $1x10^9$ cells/ml LentiKats®.

2.4. *Analytical assays*

The sizes of LentiKat® lenses with immobilized cells and the alginate microbeads were analyzed using a microscope with an accuracy of 10 mm. Cell concentrations and viabilities were determined after dissolution of lenses through heating and mixing. Yeast cell concentration was estimated with a Thoma counting chamber and the cell viability was assessed using the methylene blue-staining technique. The distribution of immobilized cells was determined by fixation of lenses and beads in 2.5% glutaraldehide and araldite and further longitudinal- and cross-sectioning (1.5 μm). Liquid samples from both growth and fermentation mediums were collected aseptically and analyzed for specific gravity, flavor volatiles, FAN, vicinal diketones, yeast cell counts, and cell viability.

RESULTS AND DISCUSSION

Growth studies have shown that a lag phase of 22 h and an exponential growth phase of 18 h, with a specific growth rate of 0.22 h^{-1}, could be distinguished on the growth curve obtained for immobilized cells in LentiKat® carriers. Released cells were detected in the medium only after 20 h of cultivation, which approximately coincided with the start of intensive proliferation of immobilized cells (Figure 4). The increase of the cell concentration in the medium was exponential, with the apparent specific growth rate of 0.43 h^{-1}, representing the combined effects of cell proliferation in the medium and cell leakage from the carriers. The overall concentration of cells in the immobilized system (within carriers and in the medium) as a function of time was compared with the growth of yeast in free cell suspension. It was found that the immobilized cells exhibited significantly longer lag and exponential phases than freely suspended cells (22 and 18 h vs. 5 and 10 h, respectively). The apparent specific growth rate in the immobilized system was almost 2-fold lower than that obtained in the free cell suspension (0.24 vs. 0.47 h^{-1}). However, the final overall cell concentration in the immobilized system was higher than the concentration achieved in the free cell suspension due to the prolonged growth in the immobilized system (Figure 5).

The growth studies in the case of alginate microbeads showed that three general phases of microbial growth can be distinguished: a short lag phase (about 4 hours), an exponential phase (about 12 hours) and a stationary phase (until the end of the experiment) (Figure 6). The highest final cell concentra-

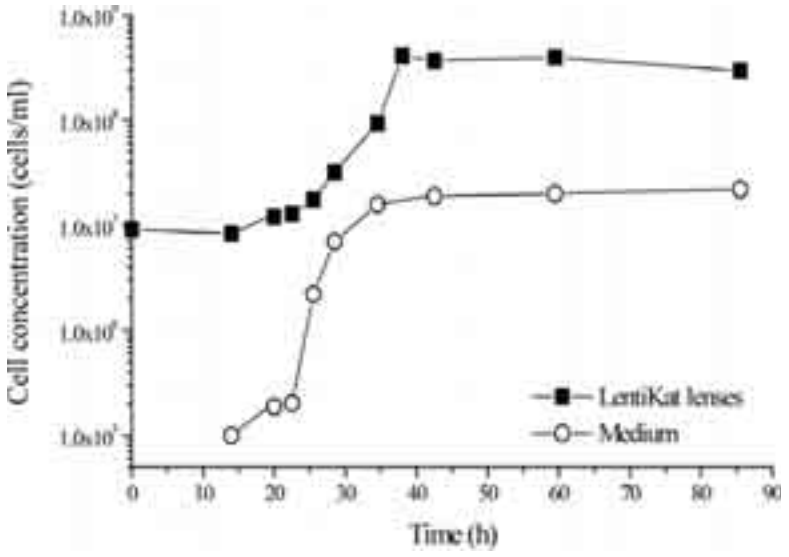

Figure 4. Growth curves in immobilized cell culture: –■– cells within LentiKat® carriers; –○– cells released into the medium.

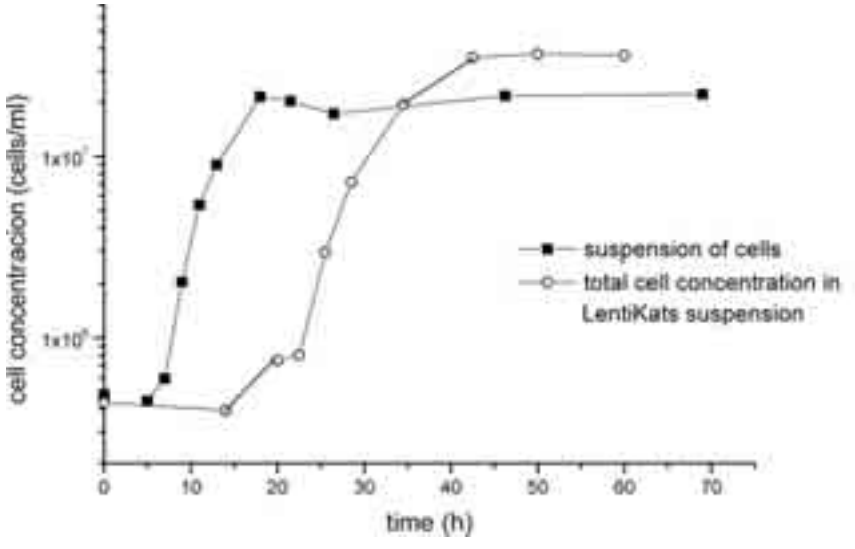

Figure 5. Comparison of cell concentrations in cell suspension and LentiKats suspension.

tion of about 2.33 x 10^9 cells/ml was found in microbeads with initial mean diameters of 0.5 and 0.6 mm.

The results of the growth studies indicated that the immobilization procedure in LentiKat® carriers had a negligible effect on cell viability and growth. The apparent specific growth rate of cells released in the medium was comparable to that of freely suspended cells, implying preserved cell vitality. In addi-

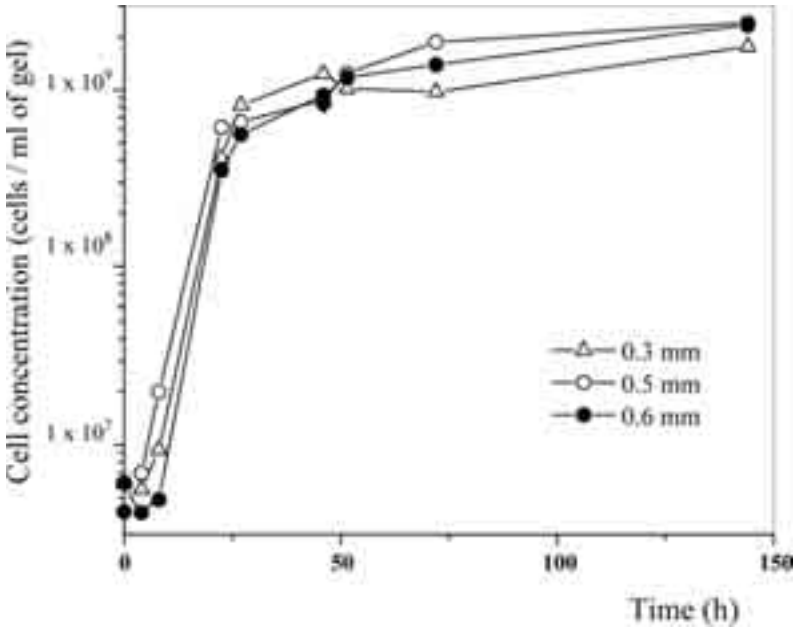

Figure 6. Yeast growth in alginate microbeads

tion, the final cell concentration achieved in LentiKat® carriers was an order of magnitude higher than the final concentration of suspended cells ($5x10^8$ cells/ml of carrier vs. $3x10^7$ cells/ml) and a little bit lower compared to the concentrations of brewer's yeast cells in Ca-alginate microbeads.

Fermentations with yeast cells, immobilized in LentiKat® carriers in shaken flasks, showed that the apparent attenuations of around 80% were achieved after two-day fermentations. The cell concentration within LentiKat® particles stabilized at the value of around $8x10^8$ cells/ml after three experimental runs, while the cell concentration in the medium was constantly increasing during fermentation runs. Constant increase of biomass production was noticed, indicating a stable functioning of the immobilized cells. High rates of biomass formation exhibited by immobilized cells could be crucial for continuous mode of application, by providing a stable source of yeast supply. The cell activity stayed constant over 4 weeks of multiple fermentations. LentiKat® particles remained intact, confirming chemical and mechanical stability. The problem noticed in these fermentation studies was significant agglomeration of particles, which resulted in the formation of clusters.

Fermentations results in the gas-lift bioreactor system were promising as well. Stable operation at gas flow rate of 240 ml/min was achieved, without agglomeration that was observed during batch fermentations in shaken flasks. The process lasted for 24 hours at relatively low solid loading (about 10%). Immobilized cells demonstrated high fermentation activity with apparent attenuation between 80 and 86%. Concentration of cells in the carrier raised from

the initial $8 \cdot 10^8$ cells/ml to $1.4 \cdot 10^9$ cells/ml, while the final concentration in the medium was $2.1 \cdot 10^7$ cells/ml.

A batch of LentiKat® particles with immobilized brewer's yeast in shaken flasks and in gas-lift bioreactors comprised of over 60 days of operating time in a 6 month period without obvious changes in shape and size. Final beers had desired sensory and analytical profiles.

CONCLUSIONS

This study has demonstrated that LentiKat® particles could be efficiently used as carriers of brewing yeast cells in beer fermentation. The results of growth studies imply that the immobilization procedure has no adverse effects on cell viability and proliferation. Although the growth phases of immobilized cells were prolonged as compared to freely suspended cells, high final cell concentrations on the order of $1x10^9$ cells/ml of LentiKats® were achieved. The immobilized cells retained such a high fermentation activity that the full attenuation in green beer was reached after 48 hours of fermentation in shaken flasks and in less than 24 hours of fermentation in the gas-lift bioreactors. Relatively low solid load was applied (10% w/v) in the gas-lift reactor, implying that even higher fermentation rates could be achieved at higher hold-ups of the solid phase. LentiKat® biocatalysts provided a stable source of yeast cells and a possibility to balance the amounts of immobilized and freely suspended cells in fermentation systems aimed at achieving high productivity and desired beer flavor.

ACKNOWLEDGEMENTS

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ТЕХНОЛОГИЈА ИМОБИЛИСАНИХ ЋЕЛИЈА У ПРОИЗВОДЊИ ПИВА — ДОСАДАШЊА ИСКУСТВА И РЕЗУЛТАТИ

Ида Ј. Лескошек-Чукаловић, Виктор А. Недовић
Институт за прехрамбену технологију, Пољопривредни факултет
Универзитета у Београду, Немањина 6, PO Box 127,
11081 Београд, Србија и Црна Гора

Резиме

Индустрија пива већ 30 година показује занимање за примену технологије имобилисаних ћелија. Најважнији разлози су: већа брзина и продуктивност ферментације у поређењу са традиционалном производњом пива на бази суспендованих ћелија, као и могућност континуалног рада. Данас је ова технологија већ конвенционално примењена у накнадном врењу и производњи нискоалкохолних и безалкохолних пива. Главно врење, међутим, још увек је остало нерешен проблем. Због своје комплексности, упркос многобројним експерименталним резултатима на лабораторијском и полуиндустријском нивоу, још није реализовано на индустријском.

У раду су приказани најважнији досад развијени процеси, носачи и биореакторски системи на бази имобилисаних ћелија, примењени у различитим фазама ферментације пива, као и најновији сопствени експериментални резултати. Испитивана је могућност примене поливинил алкохола у облику LentiKats®, као потенцијалног носача за ферментацију пива. Дати су резултати испитивања кинетике раста пивског квасца *Saccharomyces uvarum* имобилисаног на LentiKats и алгинатном носачу и упоређене са вредностима добијеним за слободне суспендоване честице. Утврђено је да поступак имобилизације на LentiKat® носачу има занемарљив утицај на виталност и раст честица. Специфична брзина раста честица које се ослобађају у медијум одговарала је вредности добијеној у случају слободних суспендованих честица. Огледи у шаржним ферментацијама у тиквицама по Ерленмајеру и гас-лифт биореактору показали су да имобилисане ћелије задржавају велику ферментативну активност. Потпуна преврелост младог пива у тиквицама по Ерленмајеру достигнута је након 48 часова и за мање од 24 часа у гас-лифт биореактору.

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Teoharis Pavlidis, Milena Ilieva, Sonja Bencheva, Jordanka Stancheva

University of Forestry, Blvd. Kl. Ochridski, 10, Sofia 1756, Bulgaria
e-mail: mushrooms_sp@abv.bg

RESEARCHES ON WOOD-DESTROYING FUNGI DIVISION *ASCOMYCOTA*, CLASSIS *ASCOMYCETES**

ABSTRACT: Orchards aging and agrotechnical cares reduction have led to suitable development conditions of a large number of wood-destroying fungi that had never been a problem for the intensive fruit growing. This caused the necessity of their study in the main orchard regions of our country. The research was conducted from 2003 to 2005 on the basis of expeditionary-geographical method. Twelve species of wood destroying *Ascomycota* fungi have been identified. Both their parasitic activity degree and phylogenetic and ontogenetic specialization level have been defined. Species with mutual hosts — fruit or forest trees have been found. That fact makes possible the infection accumulation and transfer from forest to agricultural ecosystems which is of considerable importance for the mountain fruit growing.

KEY WORDS: division *Ascomycota*, wood-destroying fungi, fruit growing, forest plantations

Orchards aging and agrotechnical cares reduction have led to suitable development conditions of a large number of wood-destroying fungi that had never been a problem for the intensive fruit growing. This caused the necessity of their study in the main orchard regions of our country.

A great part of the phytopatogenic fungi including the parasitic ones relates to *Ascomycota* division. The most characteristic features of this division are the asci — generated after the mating. They are positioned either directly on mycelium or gathered in special fruit bodies.

MATERIALS AND METHODS

The research has been conducted during the period 2003—2005 by following the expeditionary-geographical method. The study was into orchards

* The paper was presented at the first scientific meeting MYCOLOGY, MYCOTOXICOLOGY AND MYCOSES held from 20—22 April 2005 in Novi Sad.

and the situated next to them forest-tree species and bushes located in woods or parks. The research was carried out in several regions in Bulgaria — Sofia, Plovdiv, Pazardzik, Lovech etc., as well as in Greece — Thessaloniki, Ioanina, Serres, Kozani, Veria etc.

Special attention was paid to trees in bad physiological condition. The found fruit bodies were identified on the spot or in laboratory conditions. The identification guides of Laessoe (2000), Lincoff (2000), Κωνσταντινιδης (2002), Garnweidner (1996), Dermek (1979), Svrček, Vančura (1983), were used.

In this research the following indexes which indicate the host-tree healthy condition have been registered: species, physiological condition, infected plant organ, age.

The following biologic and parasitic characteristics of the wood-destroying fungi: phylogenetic, organotropic and age specialization have been analyzed as indicators of their parasitic activity degree.

Spore prints for spores microscopic analysis have been taken for the fresh found fruit bodies.

RESULTS AND DISCUSSION

In the research process, on the basis of 24 samples, 12 fungi species of *Ascomycota* division have been identified which had damaged 10 different hosts — 4 fruit-trees and 6 forest-trees. Those twelve fungi cause infections or saprophytic wood rot on of fruit or forest trees and belong to 4 orders (Table 1).

Table 1. Classification of wood-destroying *Ascomycota* fungi

Division *ASCOMYCOTA*	Order *Hypocreales*
	Nectria cinnabarina Fr. (Tode) Wint
	Order *Leotiales*
	Ascocoryne sarcoides Groves & Wilson
	Bisporella citrina Korf & Karpenter
	Dasyscyphus niveus (Hedw. ex. Fr.) Sacc
	Order *Pezizales*
	Distinct perlata (Fr.) Fries
	Scutellinia scutellata (Fr.) Lambotte
Classis *ASCOMYCETES*	Order *Xylariales*
	Hypoxylon fragiforme (Pers. ex. Fr.) Kickx
	Hypoxylon nummularia (Bull. ex. Fr)
	Physalospora obtusa (Schweitz) Cooke
	Ustulina deusta (Fr.) Petrak
	Xylaria hypoxylon (L. ex. Hook.) Grev
	Xylaria polymorpha (Pers. ex. Mer.) Grev

The most frequent wood-destroying *Ascomycota* fungi among the identified ones are: *Nectria cinnabarina* (20.8%), *Hypoxylon fragiforme* (20.8%), *Ascocoryne sarcoides* (16.8%).

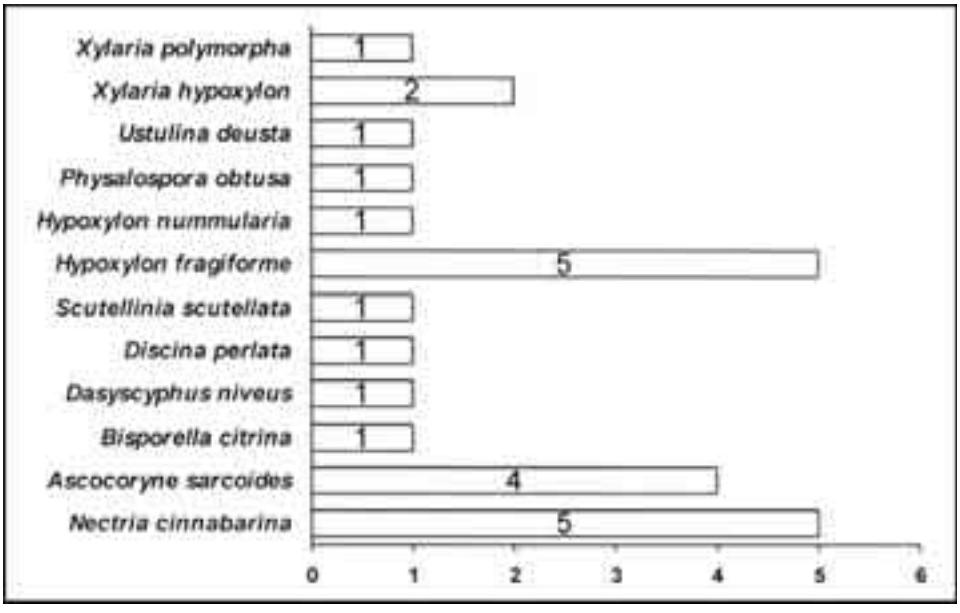

Fig. 1 Quantity of the identified species of wood-destroying *Ascomycota* fungi

In dependence with their parasitic activity level (the ability to attack living or dead organic matter) the identified wood-destroying fungi belong to three groups:

— Parasitic species which attack living trees (16,7%). The following fungi are with the highest parasitic activity level: *Nectria cinnabarina* and *Physalospora obtusa*.

— Fungi with mixed type of parasitic activity (25%). They are capable of causing pathological changes in host-trees that are weak or in bad physiologic condition, as well as saprotrophyc dead wood rot. The species: *Hypoxylon fragiforme, Hypoxylon nummularia* and *Ustulina deusta* show mixed type of parasitic activity.

— Saprotrophyc fungi which mineralize dead wood and take part in the biological energy and matter rotation (58.3%). The species: *Ascocoryne sarcoides, Bisporella citrina, Dasyscyphus niveus, Piscina perlata, Scutellinia scutellata, Xylaria hypoxylon* and *Xylaria polymorpha* are saprotrophyc fungi (Fig. 2).

The most frequent host-fruit-trees of *Ascomycota* wood-destroying fungi are: apple (33%), walnut (33%), cherry (17%) and morello (17%). That can be explained by their higher sensibility towards wood-destroying fungi and with the fact that they are cultivated in large numbers in Bulgaria (fig. 3).

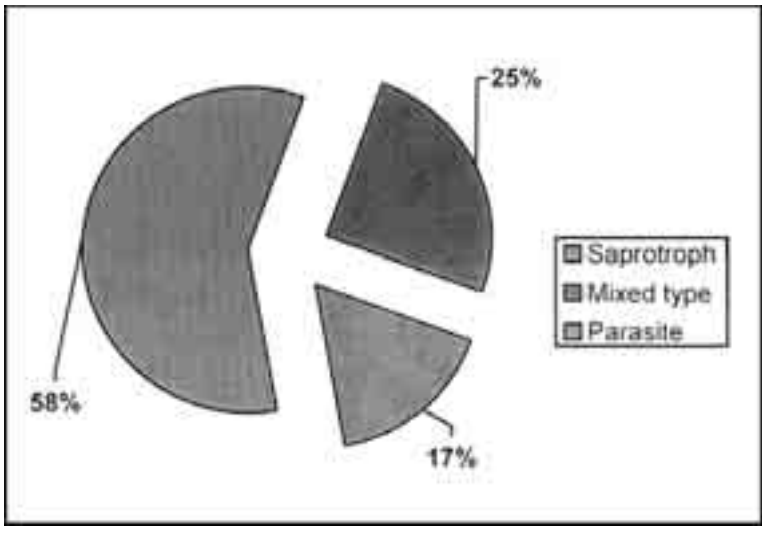

Fig 2. Valuation of the parasitic activity of wood-destroying *Ascomycota* fungi

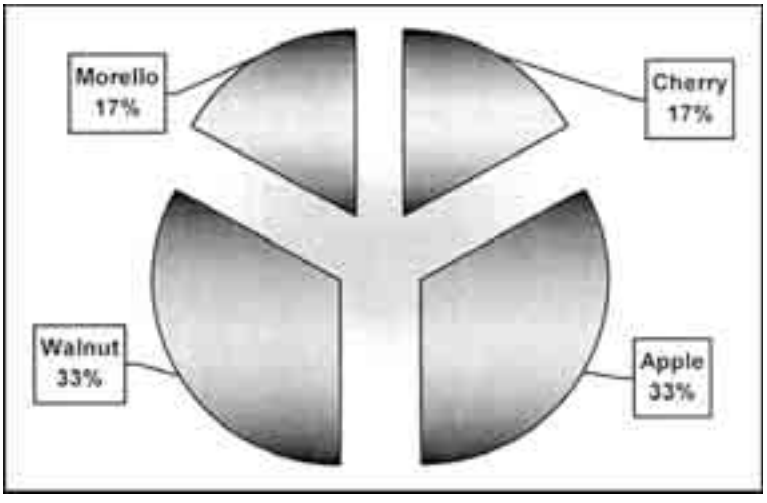

Fig. 3 Fruit cultures-hosts of wood-destroying *Ascomycota* fungi

The most frequent hosts of wood-destroying fungi among forest trees are: oak (22%), hornbeam (22%), beech (11%), fir (11%), aspen (6%), alder (6%) and other species (22%) (Fig. 4).

The identified wood-destroying fungi are able to develop on a large range of host-trees, both on deciduous and coniferous trees.

Almost perfect match is observed when comparing the literature data about the phylogenetic specialization of wood-destroying fungi and the data from our research. Species with the lowest level of phylogenetic specialization are: *Nectria cinnabarina* and *Physalospora obtusa*.

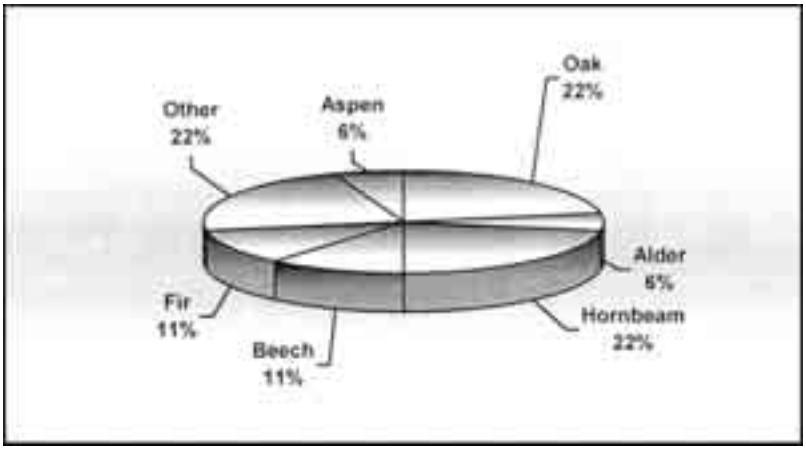

Fig. 4. Host-forest-tree species — hosts of wood-destroying *Ascomycota* fungi

Wood-destroying fungi which are capable of colonizing fruit and forest trees at the same time are dangerous for the mountain fruit-growing (Tab. 2).

Table. 2. Wood-destroying fungi which infest fruit and forest trees simultaneously

Name of wood-destroying fungi	Hosts-trees of wood-destroying fungi according to literature data	Hosts-trees of wood-destroying fungi according to the present research
Ustulina deusta	beech	beech
Bisporella citrina	oak and beech	oak
Hypoxylon nummularia	oak and beech	oak
Nectria cinnabarina	apple, pear	walnut, cherry, apple, alder
Physalospora obtusa	all fruit trees	walnut
Hypoxylon fragiforme	beech and hornbeam	beech, hornbeam, aspen and morello
Discina perlata	fir	fir

It is possible large quantity of infection to be accumulated in the forest ecosystems and transferred to the agricultural ones thus causing a massive scale attack and damage, provided the fungi development conditions are favorable.

CONCLUSION

As a result of the conducted research into wood-destroying fungi of *Ascomycota* division, the following inferences could be drawn:

1. The species: *Nectria cinnabarina, Hypoxylon fragiforme* and *Ascocoryne sarcoidesare* are the most spread ones.

2. The species: *Nectria cinnabarina* and *Physalospora obtusa* show the highest parasitic activity level. *Hypoxylon fragiforme, Hypoxylon nummularia* and *Ustulina deusta* shown mixed parasitic activity type. Saprotrophyc species

are: *Ascocoryne sarcoides, Bisporella citrina, Dasyscyphus niveus, Discina perlata, Scutellinia scutellata, Xylaria hypoxylon* and *Xylaria polymorpha*.

3. The species: *Ustulina deusta, Bisporella citrina, Hypoxylon nummularia, Hypoxylon fragiforme* and *Discina perlata* show phylogenetic specialization level. Wood-destroying fungi which can be found on large number of hosts-trees are: *Nectria cinnabarina* and *Physalospora obtusa*.

The widespread wood-destroying fungi of *Ascomycota* division represent a constant infection danger among fruit trees. The limitation measures of damage caused by wood-destroying fungi should work on in changes of the fruit trees cultivation technology.

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ИСПИТИВАЊЕ ГЉИВА КОЈЕ УНИШТАВАЈУ ДРВО, РАЗРЕД *ASCOMYCOTA*, КЛАСА *ASCOMYCETES*

Теохарис Павлидис, Милена Илијева, Соња Бенчева, Јорданка Станчева
Шумарски факултет, Булевар Климента Охридског 10, 1756 Софија, Бугарска

Резиме

Старење воћњака и смањење агротехничких мера довело је до погодних услова за развој великог броја гљива које уништавају дрво, а које никада нису представљале проблем за интензивно воћарство. То је изазвало потребу за њиховим проучавањем у главним воћарским регионима у нашој земљи. Истраживање је спроведено у периоду од 2003. до 2005. године на основу експедицијско-географског метода. Идентификовано је дванаест врста гљива *Ascomycota* које уништавају дрво. Дефинисан је и њихов степен паразитске активности, као и ниво филогенетске и онтогенетске специјализације. Пронађене су врсте које имају заједничке домаћине — воћке или шумско дрвеће. Та чињеница омогућује акумулирање инфекције и преношење из шума у пољопривредне екосистеме, што је од великог значаја за воћарство.

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Milan N. Matavulj, Nebojša Vulikić, Igor Gojković, Maja A. Karaman

Department of Biology and Ecology, Faculty of Sciences,
University of Novi Sad, Trg D. Obradovića 2,
YU — 21000 Novi Sad, Serbia and Montenegro
e-mail: matavuly@ib.ns.ac.yu

CONDITIONALLY PATHOGENIC FUNGI IN RECREATIONAL WATERS*

ABSTRACT: The improvement of health and life conditions depends on various environmental factors. The exposition to organic and inorganic pollutants, as well as to the broad spectar of microorganisms is one of these factors. Medically important fungi have been increasing their number recently, especially in urban and in recreational zones. Some of them, first of all molds and yeasts, are involved by different means in causing more or less serious deseases of man and animals. Frequency of alergic symptomes and human mycotic lesions increased significantly during last decades. Such phenomena have provoked more scientific attention recently.

According to the available literature data, micro-fungi, causing mycoses and „environmental" fungi too, could be considered as an important factor of health risk, being neglected and underestimated so far, especially in analyses of safe use of recreational waters and surrounding areas, among them swimming pools, river and sea beaches. On the basis of such statement there arises conclusion that water and ground of recreational zones could serve as vectors in transmission pathways of potentially or conditionally pathogenic fungi, being dangerous especially for immunocompromised individuals, which suggests inclusion of qualitative and quantitative composition of fungal community into a continual monitoring of hygienic status of recreational zones.

KEY WORDS: Fungi, recreational waters, health risk, mycoses, monitoring

INTRODUCTION

During the XXI century, the significant increase of incidence of infections caused by so-called „environmental" fungi has been anticipated and the fact is that ecological microbiologists are more familiar with this group of fungi than clinical ones, especially not acquainted with them to the extent which the seriousness of this problem deserves (K o o n t z, 1998).

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The incorrect opinions considering human and mammal parasitic fungi are still widely distributed, especially regarding the thought that relatively small number of fungi parasitize on Vertebrates and that these so-called „medicinal fungi" represent special group of Mycota whose confusing nomenclature and taxonomy additionally complicate their study. Nevertheless, before discussing such attitudes, it would be necessary to remind oneself that in everyday terminology use one should distinguish between the term „parasitic" and „pathogenic", where as a pathogenic the parasite who could cause a disease of the host species is characterised? Also, one can speculate that every parasite in certain circumstances and under certain conditions could become pathogenic? Recent clinical position of synonymizing these two terms have been formed due to the fact that the majority of parasites could cause more or less serious diseases.

Most of our knowledge about virulent determinants of pathogenic and potentially pathogenic fungi comes from an infected host (person), mainly from experimental animal models and recently from „*in vitro*" studies of cell cultures (M e n d e s - G i a n n i n i, 2000). Fungi represent intra and/or extracellular parasites, and parasite phenomenon depends on complementary surface molecules. It has been characterised as event of cohabitation where fungi, creating stable conditions for their survival, recognize specific tissue of host as an attractant. Infection in most cases appears with mechanic injuries and unadequate dressed wounds which become secondarily infected by microorganisms that are present in water.

It is also important to remind ourself that besides parasitic, fungi might cause health disorder in humans in some other ways. If they are ingested, they might act like poison and in contact with, in some way sensitive persons, they might cause allergic reaction (A i n s w o r t h, 1968).

Inoculation of infective agents is possible superficially, by dermic way (penetrated injuries, lacerations, being pricked when swimming); by ingestion or inhalation. Very few data considering the quantity of absorbed water during „typical" exposition to recreational waters are avaliable. The most often used standard is 100 ml per day (H a a s, 1983). Depending on the depth of inoculation and the sort of microorganisms that are inoculated, serious infections of tissues are possible: deformities and loss of function and even systematic infections. Piodermiae, eye and ear infections, as well as urogenital tract infections, are possible even without previous mechanic injury, especially in the parts where wastewater has been mixed with water that is used for recreational activities.

Certain kinds of organic dust when being inhalated do not cause visible damage, and others cause clinic symptoms in three ways:

1. Acting as allergens (complete antigens) cause sensibility and cause alergic alveolitis (especially in case of uneffective alveolar macrofag);
2. Cause direct toxic or mechanic irritations according to the type of „irritation by strange body";
3. Directly cause lung infection (M i k o v, 1995).

Inhalated dust or fungal spores, not bigger than 5 mm in diameter, are kept on mucous membrane of upper respiratoric parts, while particles smaller

than 5 μm penetrate the lower respiratoric parts (respiratoric bronchiolae and alveolae). For diagnosis it is important to prove the connection of time and space with the source of infection. The members of genus *Aspergillus* belong to the most frequent contaminants. The illnes caused by *Aspergillus* species is characterised by inflamatoric granulomatous lesions. Allergic form of lung aspergillosis appears in predisposed atopic-astmatic persons. These persons spit out the spittle that consists of eozinophylls, micelium of fungi, and in blood increased eozinofilia can be detected. Illness gives positive reactions on skin and precipitin against *Aspergillus fumigatus*.

Hypersensitive pneumonitis (allergic alveolitis) is lung illness which is in most cases caused by inhalated thermophyllic actinomycets and fungi. Moreno—Ancillo et al. (1997) report that they have noticed favourable conditions for the growth of fungi in closed warm swimming pools that were visited by their patients. To determinate possible etiologic agents, cultures from several parts of the swimming pool have been isolated. These isolates have shown significant growth of thermophyllic mycobiota where *Neurospora, Aspergillus* and *Pullularia* species have been isolated from samples.

Development of quality of life and health of people is complex function of different conditions of living environment. Here, among others, exposure to organic and inorganic pollutants as well as to the wide spectrum of microorganisms belongs too. The frequency of presence of medically important fungi has increased in urban and recreative zones. Some fungi, first of all yeasts and molds, are included by different ways in causing pathologic alterations in human and animal organisms. Allergenic symptoms and human mycotic lesions as onychomycosis for example, have been increasing in the last decade (Mendes et al., 2000). The same authors the increasing presence of potentially pathogenic fungi on the beaches of Portugal explain as the consequence of increased quantities of organic waste-materials of anthropogenic origin.

Fungal infections on humans and animals, so called *mycoses*, may be classified according to the part of the body on which they parasitize, to „cutaneous", „subcutaneous", „deep" or „systemic" ones, etc. This kind of classification, mainly based on medical mycologic tests make it easier to experts to come to realiable diagnosis. However, this kind of classification is not correlated with the types of parasitic fungi, especially from taxonomic point of view.

Among endogenous mycoses, mainly candidiases are involved (caused by *Candida albicans*), while exogenous mycoses involve coccidiomycosis (caused by fungus *Coccidioides immitis*), adiaspiromycosis (*Emmonsia* species), histoplasmosis (*Histoplasma capsulatum*, imperfect form of *Gymnoascus demonbreanii* and other systemic mycoses) the cause of which might be *Cryptococcus neoformans* (criptococosis), *Aspergillus fumigatus* and others (aspergillosis), *Sporothrix schenckii* (sporotrixosis), etc. (Ainsworth, 1968).

Since fungi may cause human and animal illnesses, they are the subject of studies of both mycology and medicine (pathology). As parasites on humans and animals, fungi may attack skin and keratinised tissues: nails, fur, hair, but also inner organs (organs for digestion, lungs, brain and so on). In the first case they are superficial parasites, very often opportunistic ones, with certain localisation of pathologic processes that they cause. In other case fungi

may cause systemic or deep mycoses, apscessee in different organs, leukemia, endocarditis and other illnesses. Clinical symptoms vary very much and range from small surface nodullae on hair and skin escharification on which spores of the fungus may be found, as well as deep inner illnesses which may have fatal result (M u n t a ñ o l a - C v e t k o v i ć, 1987).

Human and animal mycoses are rare and less dangerous than those on plants. They mostly appear on weakened and immunocompromised persons, so they could be considered as opportunistic infective agents.

To be potentially human pathogenic organisms, fungi do not have to be direct infective agents. They may cause allergic and hypersensitive reactions. Spores that in smaller and higher concentrations hang in the air, especially in the dust of contaminated areas, are important as „inhalation" allergens. Members of many families of fungi especially of the *Aspergillus, Penicillium, Alternaria, Cladosporium*, and *Mucor* genera, in their spores contain substances that can cause irritative syndroms or sensibilization.

Prerequisite of pathogenesis of many systemic invasion of fungi on humans and animals is incorporation of fungi into phagocytes. This way the fungus provides the habitat, where occurs *in vivo* transformation of mycelial into single-cell (yeast-like) round forms, the fungal answer to the defensive mechanisms of a host organism and at the same time it is the first step of penetration of fungus into cells and tissue of a host organism. This kind of fast morphological adaptation to intracellular habitat is characteristic for human pathogens such as: *Histoplasma capsulatum, Cryptococcus neoformans, Coccoidiodes immitis* and others.

Hyphae of some pathogenic members of *Zygomycotina* and of the genus *Aspergillus* keep the mycelial form when penetrate into host tissue, using specific ways of adaptation to the new environment. Inhalated spores of *Aspergillus fumigatus* after phagocytosis germinate into short, wide and bronched hyphae (ø 5—10 mm, unlike the normal of ø 3—5 mm), known as actinomycetoid that may be found with chronic aspergillosis. In infections caused by the members of *Mucor* and *Rhizopus* genera it can be noticed the increase of volume and thickness of inner wall of hyphae as an attempt of adaptation to environmental antagonism. In the last stadia of some animal illnesses hyphae recover normal vegetative mycelial form which testify about overcome resistance of the host tissue (M u n t a ñ o l a - C v e t k o v i ć, 1987).

LITERATURE REVIEW

Besides other microorganisms from the Mediterranean beaches and surfaces of swimming pools, fungal species have been isolated, too. There are a great number of micro-fungi, potential or conditional pathogens that may be contacted via beach sand, but there is no epidemiological evidence about transmission of pathogens by this way (EOS/DRAFT/98.14, 1998, WHO, 2000).

The widespread use of sea- and other natural surface waters for recreational purposes in recent years has brought to the question a problem of exposure to potential risk of health of bathers, swimmers and people who do some other

ways of recreation on water. Bacterial indicators are widely used in estimation of presence of potential pathogen in water for recreation and in the soil as a part of recreational zones. However, bacterial indicators, especially faecal coliforms, indicate only indirectly the possible presence of some potentially pathogenic fungi, first of all the presence of some kinds of yeasts. On the basis of investigation of 1576 samples from six recreational beaches in Israel, Sheinman et al. (2000) have concluded that only 4,5% of samples contained faecal coliforms in quantities not allowed by the regulative. Yeasts and molds were present in great number (91%) of samples. From 44 identified species, 15% belonged to the genus of *Rhodotorula*, 12,5% to *Candida humicola* and 12,3% to the *Candida albicans* species. Most of mold isolates belonged to the *Aspergillus* and *Penicillium* genera. Since among members of the *Candida* and *Aspergillus* genera exist species that are considered to be conditionally pathogenic, these authors recommend obligatory inclusion of „mycotic" parametres as additional indicators in estimation of relative safe use of recreational waters.

The similar results published Arvantidou et al. (2000) who, investigating 197 samples of sea water during the summer season, found 100% samples contaminated by particles of filamentous fungi, while 15% samples were contaminated by yeats. Mycelial fungi belonged to the genera: *Penicillium* (isolated from 135 samples), *Aspergillus* (isolated from 113 samples) and *Alternaria* (found in 47 samples), while yeast-like *Candida* species have been found in 8 samples. The number of yeasts was found to be in significant correlation with the number of total coliform bacteria. Between the filamentous fungi quantity and number of bacterial indicators of organic pollution, such a correlation has not been found. On the basis of these facts the authors have concluded that the sea water should be considered as a potential vector in transmission ways of conditionally pathogenic fungi, especially for immunodeficient persons, consequantly recommending the obligatory continuous monitoring from this point of view of sanitary conditions of recreational zones.

In the frame of epidemiologic investigations done on two beaches in the areas of Malaga in Spain, it has been found that indicators of faecal pollution show highly significant coefficient of regression especially with the presence of dermatomycotic fungi. Only a number of *Escherichia coli* was found to be in positive correlation with the number of *Candida albicans*. On other beaches the quantity of faecal *Streptococae* has also been found to be in high positive correlation with dermatomycotic population. Again coliforms have been found to be in the most obvious correlation with *Candida albicans* (Borrego et al. 1991).

The investigations of the beaches of Portugal seashore (Souss, 1990) revealed the presence of dermatomycota (here the traditional inadequate term — „*dermatophyta*" has been put out of usage since fungi are not plants and suffix -phyta would wrongly direct to their belonging to the plant kingdom) in 42% analysed samples of sand beaches. Most frequently present dermatomycota were: *Trichophyton mentagrophytes, Trichophyton rubrum* and *Microsporum nanum*, all of them isolated from sand areas heavily loaded by organic waste. Saprobic (not „sapro*phytic*") fungi such as *Aspergillus candidus, Asper-*

gillus ochraceus and *Aspergillus fumigatus*, have been isolated mostly from flooded and tidal areas (Izquierdo et al., 1986).

Candida albicans and other members of the family *Candida* have been isolated from the samples of soil of sand beaches in the south of France (Bernard et al., 1988). The same investigations have confirmed the presence of 8 keratinophilic and 11 nonkeratinophilic species of potentially pathogenic fungi. Izquierdo et al. (1986) have isolated 16 species from sand from the beach of north Spanish Mediterranean seashore, some of them belonging to the potentially pathogenic fungi. Most of the isolates belonged to the *Penicillium, Aspergillus* and *Cladosporium* genera.

Ghinsberg et al. (1994) have isolated fungi from all samples of sand from beaches, but not from sea water at the same localities. Boiron et al. (1983) have studied all kinds of fungi in the sea water and sand from the beach in the same area and come to the conclusion about similar qualitative composition of bacteria in the sand and sea water. They recorded the absence of *Candida albicans* and presence of yeasts exclusively of the marine origin. The isolated fungi belonged to: *Candida tropicalis, Candida parapsilosis, Candida langeronii, Candida guilliermondii, Trichosporon cutaneum* and *Torulopsis* sp. The most frequent species found in sand of sea beaches in another study of Spanish seashore belonged to the genera: *Penicillium, Aspergillus, Cladosporium, Alternaria, Mucor, Monilia, Cephalosporium, Verticillium* and *Chrysosporium* (Roses Codinach et al., 1988). The absence or low incidence of *Candida allbicans* species have been reported by other authors too (Roses Codinach et al., 1988; Figueras et al., 1992).

Quantitive structure of fungal population in 180 samples collected from 42 Spanish Mediterranean beaches ranged to the several hundred thousand cfu/g (colony forming units). The most frequently isolated species belonged to the genera of *Penicillium, Cladosporium, Aspergillus, Acremonium, Alternaria* and *Fusarium* (Larrondo and Calvo, 1989). The results of investigations performed in the region of Atica in Greece, revealed the following qualitative composition of fungi: *Candida albicans, C. crusei, C. tropicalis, C. guilliermondii, C. rugosa, Pitirosporum orbiculare, Fusarium* sp., *Penicillium* spp., *Mucor* sp., *Helminthosporium* sp. and *Aspergillus niger* (Papadakis et al., 1997). *Candida albicans* and other species from the same genus, as well as species from the genus *Fusarium* and the species *Pitirosporum orbiculare*, represented pathogenic or potentially pathogenic community. Mendes et al., (2000) investigating 42 Portugal beaches, flooded, not flooded, intermedial, and area flooded only during high tide find frequent presence of filamentous fungi from the *Penicillium, Aspergillus, Acremonium, Fusarium, Cladosporium* and *Rhizopus* genera, on all investigated beaches and in all three studied areas. Yeast-like fungi, such as: *Candida, Scopulariopsis, Trichophyton* and *Crysosporium*, were found on only few beaches, in more frequent flooded zones and with the frequent presence of people, especially in July and August. Qualitative and quantitative composition of fungi and characteristics of distribution were similar in all studied beaches. The authors conclude that fungi are good indicators of pollution of beaches by organic waste from the consumers of the-

se recreational zones, as well as by the waste brought by the tidal wave in recreational zones.

B o i r o n et al. (1983) when examining yeast of medical importance, in sand and in sea water of „Sainte-Anne" beach in Guadalupa, isolated yeast belonging to the genera: *Candida*, *Torulopsis* and *Trichosporon*. B u c h a l o et al. (1998) isolated three species of filamentous fungi from the surface water of the Dead sea: *Gymnascella marismortui* (*Ascomycotina*), which is described as a new species, *Ulocladium chlamydosporum* and *Penicillium westlingii* (*Deuteromycotina*). The isolated cultures could not grow on agar without salt, which suggests the adaptation of fungi to the hyper-saline conditions in that sea.

In vitro studies of human pathogenic fungi from samples from the beaches of Hawaii have been done by A n d e r s o n (1979). On this occasion, 4 pathogenic fungi are isolated: *Candida albicans, Trichosporon cutaneum, Microsporum gypseum* and *Trichophyton mentagrophytes.* During experimental simulations of conditions that exist on beaches, all species survived for six months, and it shows that they can be the source of infection during a significantly long period of time. Watering and drying sand alternatively caused the shorter period of surviving of all species except *Microsporum gypseum*. The increasing of temperature resulted in general shorter period of survival; 45°C was inhibitory temperature, with exception of *Trichosporon cutaneum*, which survived that temperature level for almost 6 months. The level of salt did not influence the survival of this fungus.

Microbiological community of 108 samples of water of 6 swimming pools and 3 lakes with beaches has been examined to evaluate the role of recreational waters as possible source of human illnesses in the areas of Araraquara in Brazil (F a l c a o et al., 1993). In this study *Candida albicans*, yeasts and other dermatomycota were isolated. This study has shown that recreational waters used by the population of Araraquara can be contaminated with potentially pathogenic microorganisms and could be the source of infection.

When watching 4 recreational beaches on the lake Ontario, S h e r r y et al. (1979) have come to the conclusion that opportunistic pathogen *Candida albicans* appears in water near beaches due to the faecal contamination of water. The highest number has been recorded during July and August, what was in positive correlation with the highest number of consumers of recreational zones in the course of these months.

Epidermophyton floccosum and species belonging to the genus *Trichophyton* cause superficial fungal infections of hair, nails and skin. Infection of the skin of feet, mostly among toes, so called *Tinea pedis* (A h o & H i r n, 1981) is characterised by symptoms that involve ulceration, holes and cuts of skin with strong scab. *Tinea pedis* can be transferred by direct contacts, and in swimming pools it usually happens by physical contact with surfaces such as floors in showers and dressing rooms that have been contaminated with infective fragments of skin (PWTAG, 1999). This fungus colonizes *stratum corneum* where optimal environmental conditions for this species exist. In vitro experiment shows that it is necessary approximately 3—4 hours for fungi to initiate the infection. This infection often happens among beach rescue squad and sports swimmers and it is considered as a relatively benign. The only so-

urce of dermatomycota in swimming pools and baths are the swimmers who are infected, so in monitoring of this kind of fungal infection and in the control of expanding of this disease, education is very important. Disinfections of swimmers' feet, wearing sandals in showers and dressing rooms as well as regular disinfections of floors and bottom of the pool could reduce the infection. People with „athletic feet" and with similar dermal infections should not use public swimming pools or baths (Al-Doory & Ramsey, 1987; Public Health Laboratory Service Spa Pools Working Group, 1994).

The investigations of micro-fungi in recreational and potentially recreational waters in our country are relatively new and rare. Maria Muntañola-Cvetković and Bosiljka Ristanović, (1977, 1980) should be considered as founders of these investigations in Serbia. They studied population of micro-fungi of South Adriatic and come to conclusion about the link of antropogenous factor and qualitative and quantitave composition of fungal community and about the importance of some species originating from the coastal soil. Some works deal with alochthonous micro-fungi in the lakes like the Savsko jezero (Ljaljević, 2000), Vlasinsko jezero (Vukojević et al., 1997) and water reservoirs Grošnica and Gruža (Ranković, 1998). As a rule, in all reports the presence of *Aspergillus, Penicillium, Alternaria, Cladosporium, Trichoderma, Mucor* and *Rhizopus* species, and other potential allergenic and fungal conditional causes of infection with immunodeficient persons, are noticed.

CONCLUSION

According to the available literature data, micro-fungi, causing mycoses and „environmental" fungi too, could be considered as important factors of health risk, being neglected and underestimated so far, especially in analyses of safe use of recreational waters and surrounding areas, among them swimming pools, river and sea beaches. On the basis of such statement there arises conclusion that water and ground of recreational zones could serve as vectors in transmission pathways of potentially or conditionally pathogenic fungi, being dangerous especially for immunocompromised individuals, which suggests the inclusion of qualitative and quantitative composition of fungal community into a continual monitoring of hygienic status of recreational zones.

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УСЛОВНО ПАТОГЕНЕ ГЉИВЕ У РЕКРЕАЦИОНИМ ВОДАМА

Милан Матавуљ, Небојша Вуликић, Игор Гојковић, Маја Караман
Департман за биологију и екологију Природно-математичког факултета
Универзитета у Новом Саду, Трг Доситеја Обрадовића 2,
21000 Нови Сад, Србија и Црна Гора

Резиме

Фреквенција присутности родова и врста гљива од медицинског интереса и значаја порасла је у последње време у нашем природном окружењу, а нарочито у урбаним и рекреационим зонама. Неке гљиве, пре свега квасци и плесни, укључене су на различите начине у изазивање патолошких стања код људи и животиња. Алергенски симптоми и хумане микотичне лезије у значајном су порасту у последњој декади. Ове појаве проузроковале су већу пажњу научне јавности последњих година, посебно из разлога што се данас схвата да је унапређење квалитета живота и здравља код људи комплексна функција различитих услова животне средине. Ту, између осталог, спада и излагање органским и неорганским полутантима и широком спектру микроорганизама.

Преглед доступних података из литературе омогућава закључак о микрогљивама као факторима ризика, који су досад били занемаривани у анализама здравствене безбедности коришћења рекреационих вода, поготову базена и речних и морских плажа. На основу оваквих налаза закључује се да вода и тле рекреационих зона треба да се посматрају као потенцијални вектори у трансмисионим путевима (потенцијално) патогених гљива, посебно за имунокомпромитоване особе. То подвлачи потребу укључења квалитативног и квантитативног састава микрогљива у континуираном мониторингу хигијенске исправности рекреационих зона.

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Milan N. Mataulj[1], *Maja A. Karaman*[1], *Igor Gojković*[2], *Svjetlana Đurđević*[3]

[1] Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Trg D. Obradovića 2, YU — 21000 Novi Sad, Serbia and Montenegro
[2] Faculty of Medicine, University of Novi Sad, Serbia and Montenegro
[3] Faculty of Sciences, University of Banja Luka, Republic of Srpska
e-mail: matavuly@ib.ns.ac.yu

LIGNICOLOUS MACROFUNGI OF THE BARDAČA FLOODPLAIN REGION*

ABSTRACT: In the frame of biodiversity investigation of the Bardača floodplain (Republic of Srpska, Bosnia), the investigation of the presence and the diversity of macrofungi of the wider Bardača region have been undertaken. The relative poor generic diversity of lignicolous macrofungi with only 21 species (11 families) representing this group has been recorded. Such a poor qualitative and also quantitative composition of this very important fungal group could be explained by heavy devastation of autochthonous plant communities, reducing them to the small number of plant associations of poor generic composition. Consequently, drastic decrease of the diversity of ecological niches as fungal habitats was caused. Even though being preliminary, our results point to the necessity of conservation and protection of recent fungal diversity but, in our opinion, not by making so-called „Red list of endangered species", which, due to the lack of information and very poor evidence on this group of organisms in the region under the consideration, are extremely unreliable and therefore disputable, but rather through the very short list of few not endangered species, conditionally called „White list of not endangered fungal species", if such species recently exist at all.

KEY WORDS: lignicolous fungi, evidence, Bardača floodplain, Republic of Srpska, Bosnia

INTRODUCTION

The investigations of macromycetes of the Bardača floodplain region (Republic of Srpska, Bosnia) have been neglected so far. Data considering the qualitative and quantitative composition of these, first of all very important reducers of organic matter are lacking, as well as the data about their significant role as important elements in the entire chain of the nutrition in natural envi-

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ronments. The fungi in general, and consequently macrofungi too, become recently a group of organisms of great biotechnological interest as potential producers of various biologically active agents (Matavulj et al., 1993a, b; 1996).

The history of mycological research in former Austro-Hungarian Kingdom is being coupled with the name of Austrian botanist Schulzer von Muggenburg (1802—1892), who had already published a paper under the title „Systematische Aufzählung der Schwämme Ungarns, Slavoniens und des Banats, welche diese Länder mit anderen gemein haben" in 1858 in the journal „Verh. zool. bot. Ges. Wien", 7: 127—152 (Tortić, 1980).

Next reports consider the results of the research of the Laboratory for mycology and lichenology (Institute of Biology, Faculty of Natural Sciences, University of Novi Sad) dealing with the same problematic of the river Tamish bank region, and Fruška Gora and Vrshachke Planine mountains (Jarić et al., 1998, Matavulj et al., 1989; Matavulj and Bokorov, 1990).

The aim of this research is collecting necessary informations in this field in order to form a basis for conservation and improvement of existing condition of natural environments. Fungi are pointed as especially endangered group of organisms of Europe (Arnold and de Vries, 1993); Ing, 1993; Ivančević, 1995). Since for the Bardača floodplain region the data about macrofungi are lacking, and some parts of this region belong to planned nature reserve, we found worthy and interesting to start with the evidence of fungal species as the beginning of more systematic and more detailed investigations of the presence, biology, ecology and conservation of these significant and endangered organisms.

MATERIAL AND METHODS

Systematic mycological investigations of the Bardača floodplain region of the northern sector of the Republic of Srpska (Bosnia and Herzegovina) were done during the 2002—2004 period of time. Ten localities (sampling sites) were chosen for the collection of samples: 1) Oak wood nearby Bardača motel (100—300 m north of motel); 2) Oak and hornbeam wood, nearby the Faculty of Sciences of the Banja Luka University Research station; 3) The park with planted allochthonous wood species surrounding the Bardača motel; 4) Trees and bush along the Matura river; 5) Trees and bush along the Stublaya swamp; 6) Trees and bush along the Brzaja spring; 7) Trees and bush along the Vrbas River; 8) Utvaj region and trees along the Sava River; 9) Farms and orchards between the Stublaja and Brzaja springs; 10) Wett meadows along the productive basins of the Bardača fishponds.

Fungi were identified on the basis of both morphological and anatomical properties of fruit bodies and according to specific chemical reactions using modern keys (Ainsworth et al., 1973; Bon, 1988; Božac, 1989; Cetto, 1979; Focht, 1979; Hermann, 1990; Moser, 1978; Phillips, 1983).

RESULTS AND DISCUSSION

Results of recording of fungal species in the Bardača floodplain region are shown in Table 1 containing the list of lignicolous fungi. Most of them can be found during the whole year, regardless of the season, except *Flammulina velutipes* appearing usually during the late winter and early spring seasons, *Coprinus micaceus* and *Coprinus disseminatus*, which do not grow only during the winter, and *Pholliota cerifera*, which belongs to group appearing during summer and autumn seasons.

Out of 21 evidenced lignicolous macrofungi, to the *Polyporaceae* belonged 6 species, followed by 4 members of fam. *Tricholomataceae*, two of them belonged to fam. *Ganodermataceae* and two to *Coprinaceae*, and by one species fam. *Schizophyllaceae*, *Strophariaceae, Auriculariaceae, Hypocreaceae, Tremellaceae, Hymenochaetaceae* and *Lycoperdaceae.*

Considering the poor qualitative composition of host plants, substrates consisting mainly from six tree species: *Quercus* spp., *Crategus monogina*, *Salix alba, Populus nigra* and *Acer campestre*, it was not surprising relatively poor generic composition of lignicolous macrofungi of the investigated region.

Since the fungi are one of the most important group of organisms playing the most significant role in organic matter reduction and mineralization in natural environments, it is necessary to undertake measures for conservation of existing fungal genofond and creating conditions for comeback of species, whose withdrawal from this region was caused by impoverishing of ecological conditions for their growth.

For the conservation of existing fungal genofond and its improvement it is necessary to conserve and where it is possible to reconstruct and improve autochthonous, even rudimentary present plant associations, in order to provide substrates and ecological niches for fungal appearance.

We also find promotion of so-called „Red lists of endangered fungal species" (I v a n č e v i ć, 1995, 1996) in natural environments such as these existing in South-eastern Europe, drastically devastated by anthropogenic monoculture introduction, not to be justified, due to very limited knowledge regarding this group of organisms and due to the very distinct lack of informations about the both, former and recent presence or absence of fungal species at this territory and for one longer period of time.

Table 1. Generic composition of the lignicolous macrofungi of the Bardacha floodplain region

Species	Familia	Substrate	Locality
Ganoderma applanatum (Pers.) Pat.	*Ganodermataceae*	on fallen unidentified tree trunk and on the *Salix alba* tree	6)
Ganoderma lucidum (Curtis: Fr.) Karsten	*Ganodermataceae*	in the basis of an old willow stump; on fallen trunk of *Salix alba* in the canal bank region	1), 4), 6)
Auricularia auricula-judae St. Amans	*Auriculariaceae*	*Robinia pseudoacacia* fallen branches	3)

Flammulina velutipes (Curt: Fr.)	*Tricholomataceae*	in the basis of *Salix alba* living tree	6), 8)
Panellus stipticus (Bull.: Fr.) Karsten	*Tricholomataceae*	on old *Salix alba* stump	4), 6), 7)
Pleurotus ostreatus (Jack.: Fr.) Kummer	*Tricholomataceae*	on living *Salix alba* trunk	4), 7), 8)
Panus tigrinus (Bull.: Fr.) Sing.	*Tricholomataceae*	on fallen *Salix alba* trunk, in willow trunk cervice	1), 2), 9), 10)
Schizophyllum commune Fr.: Fr.	*Schizophyllaceae*	on dry fallen branch of unidentified tree; on fallen *Salix alba* trunk	1), 2), 4), 5), 7), 8), 9)
Pholiota cerifera (Karst.) Karst.	*Strophariaceae*	on living *Populus nigra* tree	7), 8)
Laetiporus sulphureus (Bull.: Fr.) Murrill	*Polyporaceae*	on living tree of *Salix alba*	4), 7), 8),
Daedaleopsis confragosa (Bol.: Fr.) J. Schroeter var. *tricolor* (Bull.) Bond.	*Polyporaceae*	on strait standing dead *Salix alba* trunk	9)
Trametes versicolor (L.: Fr.) Pilat	*Polyporaceae*	on *Salix alba* stumps; on linden fallen branch; on oak and on hornbeam stumps	1), 2), 3), 5), 7), 8), 9), 10)
Trametes hirsuta (Wulfen: Fr.) Pilat	*Polyporaceae*	on *Salix alba* stumps	2), 3), 7), 8), 9), 10)
Lenzites betulina (L.: Fr.) Fr.	*Polyporaceae*	on *Salix alba* stump	5), 6), 7), 8), 9), 10)
Fomes fomentarius (L.: Fr.) Fr.	*Polyporaceae*	on old trunk of *Populus nigra*; on living *Salix alba* treee; on living linden tree.	1), 3), 8)
Phellinus igniarius (L.: Fr.) Quel.	*Hymeno-chaetaceae*	on living *Salix alba* trees	8), 9)
Coprinus disseminatus (Pers.: Fr.) Fr.	*Coprinaceae*	in the basis of stump of *Populus euramericana*	9), 10)
Coprinus micaceus (Bull.: Fr.) Fr.	*Coprinaceae*	on almost all of rotten *Salix alba* stumps	8)
Lycoperdon pyriforme Schaeff.: Pers	*Lycoperdaceae*	in the basis of *Salix alba* rotten stump on the *Populus nigra* stump	5), 10)
Nectria cinnabarina (Tode: Fr.) Fr.	*Hypocreaceae*	on dry fallen branch of *Acer campestre* on dry fallen branch of *Crategus monogina*	3), 4), 8), 9), 10)
Tremella mesenterica Retz.: Hooker	*Tremellaceae*	on fallen branch of *Robinia pseudoacacia*	3)

Being a group of organisms the most sensitive to the anthropogenic natural changes (pollution, decrease of ecological niches diversity, excessive exploitation, the eradication by fungicides, etc.), fungi are the most endangered organisms. From this reason, on this stage of our knowledge of problems of fungal species diversity, more convenient and more justified would be establishing a (very) short „White list of not endangered" fungal species which would serve much more adequately for the protection and conservation of this extremely important link in the matter cycle and energy flow, first of all in terrestrial ecosystems.

CONCLUSION

During the 2001—2004 period of time, investigations of presence and species diversity of macrofungi in the Bardača floodplain region have been undertaken. 21 lignicolous species were recorded, representing 11 families. Relatively poor generic composition can be explained by drastic anthropogenic devastation of autochthonous plant associations and by reducing the vegetation along the fishpond banks to the small number of plant species, causing the reduction of the diversity of ecological niches for growth of fungi, as a rule highly specified for dead or living plant substrate.

Since in natural environments fungi (together with bacteria) play role of one of the most important group of mineralizators of organic matter, important link in the matter cycle and energy flow through the ecosystem, it is necessary to prevent further devastation of fungal species diversity and undertake measures for conservation of existing fungal genofond and for its diversity improvement by reconstruction at least fragmentary autochthonous plant associations which would cause the enrichment of ecological niches diversity and consequently to that enrichment of fungal species diversity.

From mycological point of view, the Bardača floodplain region is not *terra incognita* any more, but for more reliable data, further more systematic and more detailed investigations of this region should be undertaken.

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ЛИГНИКОЛНЕ МАКРОГЉИВЕ МОЧВАРНОГ РЕГИОНА БАРДАЧА

Милан Матавуљ,[1] Маја Караман,[1] Игор Гојковић,[2] Свјетлана Ђурђевић[3]
[1] Департман за биологију и екологију Природно-математичког факултета, Универзитета у Новом Саду, Србија и Црна Гора
[2] Медицински факултет Универзитета у Новом Саду, Србија и Црна Гора
[3] Природно-математички факултет Универзитета у Бањој Луци, Република Српска

Резиме

У оквиру истраживања биодиверзитета испитиване су заступљеност и разноликост лигниколних гљива ширег подручја мочварног региона Бардача (Република Српска, Босна). Констатован је релативно сиромашан генерички састав ове групе гљива са свега 21 врстом (11 породица). Сиромаштво ове изузетно важне групе примарних редуцената, како у квалитативном тако и у квантитативном смислу, могло би се објаснити изразитом девастацијом аутохтоних биљних заједница као природних станишта ових гљива, редукованих на мали број врста

биљних домаћина, супстрата за њихов развој. Редукција разноврсности еколошких ниша ових гљива проузроковала је смањење броја врста, што ће бити погоршано најновијом девастацијом (2004) фрагмената шумских заједница у оквиру рибњака Бардача. Због недостатка података, у овом случају се не може говорити о примени „црвене листе" угрожених врста, него о евентуалној краткој „белој листи" неугрожених врста, уколико такве у овом региону данас уопште постоје.

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Milica V. Ljaljević-Grbić, Jelena B. Vukojević, Jasmina M. Glamočlija, Dušica A. Janošević, Dragoljub V. Grubišić*, Jelena T. Lević***

Institute of Botany and Botanical Garden Jevremovac, Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia and Montenegro
* Institute for Biological research „Siniša Stanković", Bulevar despota Stefana 142, 11000 Belgrade, Serbia and Montenegro
** Maize Research Institute, Slobodana Bajića 1, Zemun Polje, 11000 Belgrade, Serbia and Montenegro

FUNGAL INFECTIONS OF *ADONIS VERNALIS* L. FRUITS*

ABSTRACT: Yellow pheasant´s Eye is a herbaceous plant from dry ressy areas. Owing to habitat destruction and over- collection for ornamental and medical purposes, *A. vernalis* L. has became scarce in central and south Europe. The reasons for *A. vernalis* threatened are manyfold. The low seeds germination rate is significant. According to our investigation the main cause of fruit destruction is fungal infection. From the surface of the fruits, collected in Deliblato Sands, the following micromycetes has been isolated and determinated: *Fusarium solani* (Mart.) Sacc., *Fusarium sporotrichioides* Sherb., *Alternaria* sp. and *Drechslera* sp. Histologycal analysis showed the presence of conidiomata and conidia *Phoma* sp. in the seeds.

KEY WORDS: *Adonis vernalis*, fruits, micromycetes, *Phoma* sp., seeds

INTRODUCTION

Adonis vernalis L. (Yellow pheasant's Eye) is a herbaceous perennial and a tipical stepe plant. In the middle and southwest Europe the area is disjunct with some isolated growth places in a mainly azonal habitats scattered from south-east Sweden to south-east Spain (Jalas & Suominen, 1989). Its grown places in central and south Europe are restricted to isolated grown places, but in more easterely Europe populations are increasing. Owing to habitat destruction and ower-collection for ornamental and medical purposes, *A. vernalis* has became scarse in central and south Europe.

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Within the menyfold reasons for *A. vernalis* threatened the low seed germination is very significant. *A vernalis* is an element of dense grassy places in which the seeds often have difficulties to reach open soil surface. In spite of this, regeneration by seeds does not take place each year. The seeds lose their viabillity very quickly, and no seed reserve is built up in the soil. Many seedlings die off due to soil desiccation in summer and frost in winter. An individual plant does not flower before its third or fourth year. Fertile reproduction only take place if seeds are abudant and the weather is rainy and cool in July to August, enabling the seeds to germinate immediately after maturing. Thus, vegetative growth is far more important than generative reproduction and happens by rhizomes producing new shoots each year (M e l n i k, 1998).

MATERIAL AND METHODS

The specimens for mycological and histological analysis (fruits of *Adonis vernalis*) were collected from Deliblato Sand, protected area.

Mycological analysis

The seeds were surface desinfected with 4% NaOCL and another seed samples left unthreated and than placed, in moisting chambers and on malt agar (MA) (B o o t h, 1971a). The morphologicaly different micromycetes were reisolated on selective mycological media. After the period of incubation, the fungal structures were placed on microscopic slides and stained with Lactophenol cotton blue. Reproductive structures were measured and photographed on Reichert microscope with Canon Power Shot S40. The micromycetes from seed surface were determined using B o o t h (1971b) and E l l i s (1997) identification keys.

Histological analysis

Specimens were fixed in FAA (formalin-glacial acetic acid-ethanol, 10:5: 85) at 4°C, 3 days. Fixed material was dehydrated in graded ethanol series and embedded in paraffin at 57°C. Sections (8—10 mm thick) were stained with haematoxylin and a second contrasting strain safranin (0.8%). All sections were photographed on Reichert microscope with Canon Power Shot S40.

RESULTS AND DISCUSION

A high degree of black and destroyed fruits probably caused by fungi was observed. From the surface of *A. vernalis* fruits, the following micromycetes have been isolated: *Alternaria* sp., *Drechslera* sp., *Fusarium solani* (Mart.) Sacc and *Fusarium sporotrichioides* Sherb. (Hyphomycetes, Deuteromycotina). After the seed desinfection from the seed surface it was isolated

only *Drechslera* sp. Species from genus *Alternaria* and *Drechslera* are transmitted through seeds. Some species are known as seed-borne pathogens. Their conidia colonize the seed coat during the seed developement stage and when the seed germinates, they become active (W a t a n a b e, 2002). *Fusarium solani* is one of the most ubiquitous soil fungus and a destructive plant pathogen of hundreds of hosts, causing root and fruit rots (S h a m i a m et al., 2003).

We analyzed immature fruits of *Adonis vernalis* (Fig. 1). The structure of fruits was disrupted. The longitudinally-sectioned fruits showed an absence of normal pericarp layers: egzocarp, mesocarp and endocarp. Testa (seed coat), embryo and endosperm was destroyed. We did not notice normal structure ot seed coat. The sections show only some parts of testa without visible cell layers. In addition, we observed an absence of embryo and endosperm (Fig. 1.1). The whole immature fruit with seed was full of mycelium and reproduc-

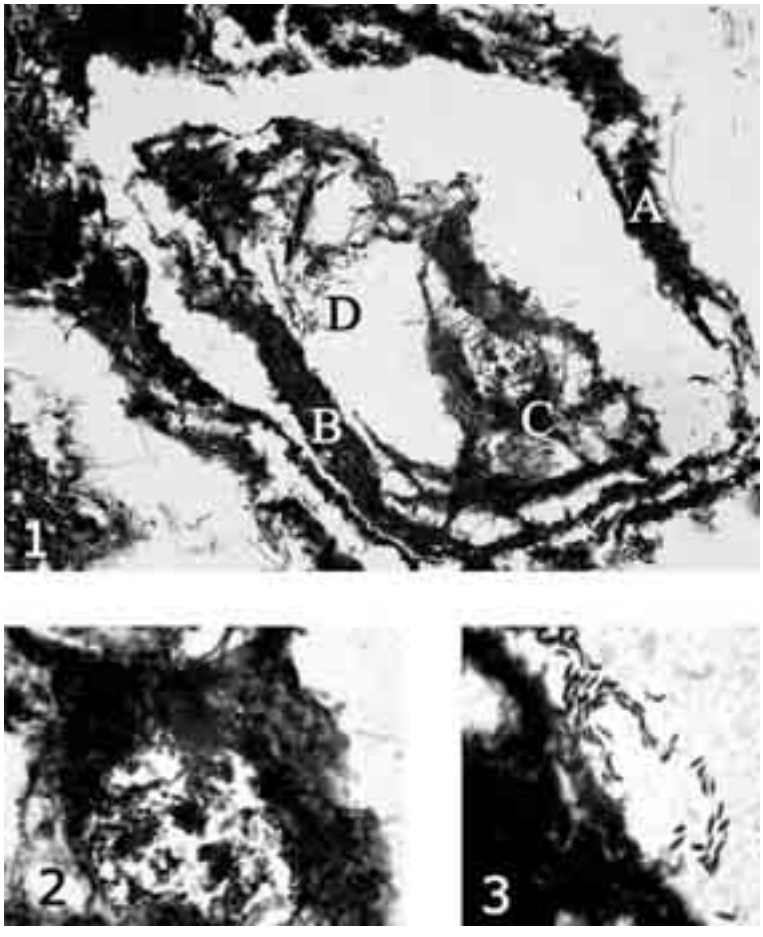

Fig. 1. The longitudinall section of infected *Adonis vernalis* fruits
Fig. 1.1. A. Destroyed pericarp; B. Destroyed testa; C. Reproductive structures of *Phoma* sp. Fig. 1.2. Conidiomata of *Phoma* sp. Fig. 1.3. Conidia of *Phoma* sp.

tive structures of fungi which was first determined as Coelomycetes. According to mycological experts from Kew Greden, England, who confirmed the identification, conidiomata and conidia belong to genus *Phoma* (Fig. 1.2 and Fig. 1.3). The investigations of developement of reproductive structures of *Phoma macdonaldi* Boerema on sunflower seeds showed a complete disintegration of the cotyledon and the picnidia arranged in rows formed in the outer layers of the parenchyma (Stajić at al., 2001).

Long-term examination of *Dianthus superbus* ssp. *superbus* on habitat Brezi in Protected Landscape area (PLA) Litovelske Pomoravi (Czech Republic) showed similar results. Three pathogenic fungi, *Alternaria dianthi, Fusarium oxisporum* and *Verticillium albo-atrum* were isolated from seeds and capsules and their negative influence on the germination and developement of young plants was proved (Mikulik et al., 2001—2002).

This results arise new questions, ideas and solutions in the concept of the threats of plant species.

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ГЉИВИЧНЕ ИНФЕКЦИЈЕ ПЛОДОВА *ADONIS VERNALIS* L.

Милица В. Љаљевић-Грбић, Јелена Б. Вукојевић, Јасмина М. Гламочлија*,
Душица А. Јаношевић, Драгољуб В. Грубишић*, Јелена Т. Левић**
Институт за ботанику и Ботаничка башта Јевремовац,
Биолошки факултет, Универзитет у Београду
* Институт за биолошка истраживања „Синиша Станковић”,
11000 Београд, Србија и Црна Гора
** Институт за кукуруз, Слободана Бајића 1, Земун поље,
11000 Београд, Србија и Црна Гора

Резиме

Гороцвет је зељаста биљка сушних предела. У централној и јужној Европи, као типично степска биљка, има ограничено распрострањење, док је у источној Европи бројност популација у опадању. У централној и јужној Европи ова биљка постаје све угроженија због претеране експлоатације од стране човека, у медицинске сврхе, као и због нарушавања њеног станишта. *A. vernalis* је као угрожена биљка укључена у црвене књиге. Један од разлога угрожености *A. vernalis* је ниска способност клијавости семена. Према нашим истраживањима главни узрок деструкције плодова је инфекција гљивама. Са површине плодова *A. vernalis*, сакупљених у Делиблатској пешчари, изоловане су и детерминисане следеће микромицете: *Fusarium solani, Fusarium sporotrichioides, Alternaria* sp., *Drechslera* sp. Хистолошки пресеци инфицираних плодова показују значајне промене: перикарп и семена су разорени мицелијом и плодоносним телима гљиве из рода *Phoma*, према мишљењу миколошких експерата (Kew Garden, Енглеска) који су потврдили идентификацију. Ови резултати доносе нова питања, идеје и решења о концепту угрожености биљних врста.

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Jelena B. Vukojević,
Milica V. Ljaljević-Grbić

Institute of Botany, Faculty of Biology, University of Belgrade,
Takovska 43, 11000 Belgrade, Serbia and Montenegro
e-mail: vjelena@bfbot.bg.ac.yu

MOULDS ON PAINTINGS*

ABSTRACT: Spores of many fungal species are present in the air. It is known that main reasons of fungal expansion in museums are inadequate relative humidity, and temperature. Regulation of these two factors can control the germination and development of moulds spores.

Isolation and determination of micromycetes from objects which are exhibited and deposited in Museum of Naive Art „Ilijanum" and in the gallery „Sava Šumanović" in Šid were done.

It was analyzed 40 samples from canvas, dyes and wooden frames with visual changes. Many species of genera *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Drechslera*, *Epicoccum*, *Penicillium*; *Rhizopus*, *Trichoderma*, *Ulocladium*, and *Wardomyces* were isolated and determined.

KEY WORLDS: moulds, museums, paintings

INTRODUCTION

Microorganisms common by attack materials such as paper, textile, wood, dyes, and leather. They form well-known brown spots on the surface of organic materials. It is known that microorganisms attacking museum objects grow fast in tropic conditions or in closed spaces with relative humidity over 70% and temperatures over 15°C. When the temperature and humidity values are low, microorganisms do not grow and the infected objects stay more or less under control.

The optimum conditions for fungal growth include a humid environment and a neutral to acidic pH with an organic nutrition source. Their development in paints may cause both aesthetic and physical degradation of the painted surface. Dust and other air components can be potental natural sources of fungi and bacteria spores. Fungal spores land on surface and grow under optimal en-

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vironmental conditions (B u s s j a e g e r, 1999). For this reason, millions of dollars are spent annually on chemicals to protect painting from microbial damage.

Moisture is the primary environmental condition, while temperature plays less important role for molds growth. Fungal spores are present in the air from 100 to over 1.000 per m^3 depending on geographic location. Moulds can appear in two forms, as spore and as mycelium. They can be transported from one surface to another by insects, humans, or air and affect the appearance and performance of paintings.

The most common fungal species found on contaminated dry paint film are *Aureobasidium*, *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium*. However, the dominant fungal species can vary depending on climate and condition of the paint film.

The aim of this investigation was the isolation and determination of mould species in paintings which can cause significant damages both in store rooms and galleries.

MATERIAL AND METHODS

The isolation and determination of micromycetes from objects which are exhibited and deposited in Museum of Naive Art „Ilijanum" and in the gallery „Sava Šumanović" in Šid were done.

Forty samples from canvas, dyes and wooden frames with visual changes were taken for mycological analysis. Samples were collected from paintings which were either deposited in museum store room or exhibited in gallery.

Samples were inoculated on malt streptomycin agar (MSA) medium (malt extract agar with 500 mg streptomycin per litar). Cultures were incubated at 25°C for 7 days. Reisolations of the formed colonies were done successively, to the selective nutrient media [potato dextrose agar (PDA), Czapek's agar (CzA) and malt extract agar (MA) (B o o t h, 1971)] using standard mycological methods: Reisolated cultures were incubated at 25°C in an incubator.

Macroscopic and microscopic characteristics of the obtained isolates were studied. Lactophenol or fuchsin acid were used for light microscopy examinations. For the identification of the fungi the following keys were used: E l l i s (1976), A i n s w o r t h et al. (1973), R a p e r and F e n n e l l (1965), R a m i r e z (1982), and W a t a n a b e (2002).

RESULTS AND DISCUSSION

Moulds species from 13 genera were isolated and determinated by mycological analysis of collected samples from paintings. Slight diference was noted between isolates from paintings surfaces and frames (Table 1). *Cladosporium* is the most abundant genus which is 4-fold more numerous then genus *Penicillium* that is the second by proportion. *Trichoderma*, *Aspergillus*, *Alternaria* and *Rhizopus* are also commonly found genera (Fig. 1). These results are in accordance with the literature data (K e c k, 1964).

Table 1. List of isolated molds species

Isolated molds species	
canvas and dyes	frames
Alternaria alternata	*Alternaria alternata*
Alternaria sp.	*Alternaria* sp.
Aspergillus candidus	*Aspergillus flavus*
Aspergillus sp.	*Aspergillus fumigatus*
Aspergillus versicolor	*Aspergillus niger*
Aureobasidium pullulans	*Cladosporium cladosporioides*
Cladosporium cladosporioides	*Cladosporium* sp.
Cladosporium sp.	*Cladosporium tenuisimum*
Cladosporiun herbarum	*Drechslera* sp.
Drechslera sp.	*Epicoccum purpurascens*
Epicoccum purpurascens	*Micelia sterilia*
Micelia sterilia	*Mycotypha microspora*
Penicillium cyclopium.	*Penicillium* sp.
Penicillium sp.	*Rhizopus stolonifer*
Rhizopus stolonifer	*Trichoderma viride*
Trichoderma viride	*Ulocladium chartarum*
Ulocladium chartarum	*Ulocladium* sp.
Ulocladium oedemansii	
Ulocladium sp.	

Species of these genera are good producers of lignocellulosic enzymes and acids which may degrade wood, paper, cardboard, cloths, dyes. Species of genera *Alternaria* and *Trichoderma* are especially destructive due to high level of lignocellulosic enzyme production. These species are very important because many objects from Museum of Naive Art „Ilijanum" were done on wood. Isolated species *Aureobasidium pullulans* is known as a potent dye and polish degrader, while species of the genus *Drechslera* are causative agents of mouldeness in museum store rooms (D i x and W e b s t e r, 1995).

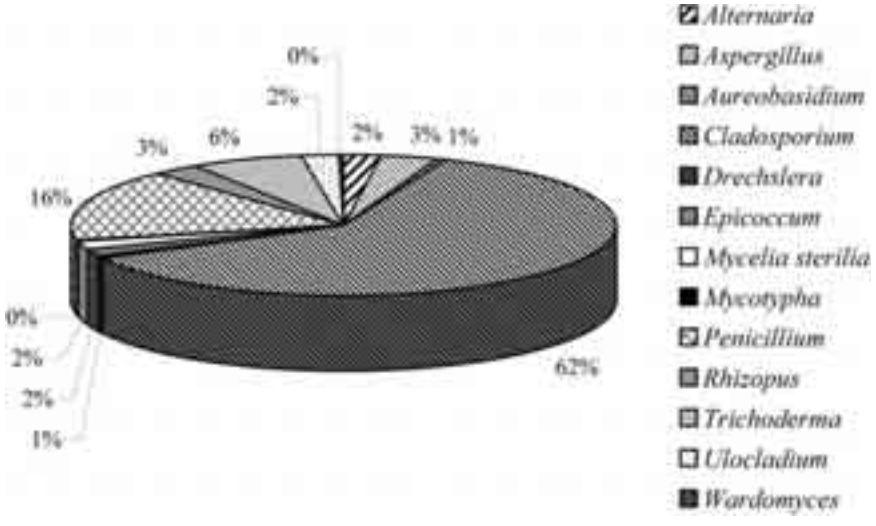

Fig. 1. Fungal genera abundant in analyzed paints

All the found species are common allergens and some of them are potential mycotoxin producers. *Cladosporium* and *Penicillium* species, which were the most abundant, are known as common causes of extrinsic asthma. Likewise, *A. alternata* is capable of producing tenuzonic acid and other toxic metabolotes which may be associated with diseases in humans and animals (C a r t e r, 1992).

It is a fact that fungal spores are present everywhere, but it is necessary to control their germination by regulation of moisture and temperature. Likewise, successful ways of protection are the usage of proper fungicides, as well as regular cleaning of museum objects, store rooms, and galleries.

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ГЉИВЕ НА УМЕТНИЧКИМ СЛИКАМА

Јелена Б. Вукојевић и Милица Љаљевић-Грбић
Институт за ботанику, Биолошки факултет, Универзитет у Београду,
Таковска 43, 11000 Београд, Србија и Црна Гора
e-mail: vjelena@bfbot.bg.ac.yu

Резиме

Споре многих врста гљива су присутне у ваздуху. Познато је да су основни узроци пренамножавања гљива у музејским просторима неадекватна релативна влажност и температура. Регулацијом ова два фактора могу се држати под контролом клијавост спора гљива и развој плесни.

Извршени су узорковање и детерминација микромицета са уметничких слика изложених и депонованих у Музеју наивне уметности „Илијанум" и у Галерији „Сава Шумановић" у Шиду.

Анализирано је 40 узорака са платна, боје и дрвених рамова са видљивим променама. Детерминисан је већи број врста микромицета из родова: *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Drechslera*, *Epicoccum*, *Penicillium*; *Rhizopus*, *Trichoderma*, *Ulocladium*, *Wardomyces*. Врсте ових родова су добри продуценти лигноцелулолитичких ензима и киселина који разграђују дрво, папир, картон, тканине, сликарске боје. Познато је да су посебно деструктивне врсте родова *Alternaria* и *Trichoderma* због високе продукције лигноцелулолитичких ензима. Велик број слика у Музеју наивне уметности „Илијанум" урађен је на дрвеној подлози па су због тога ове врсте посебно значајне. Изолована врста *Aureobasidium pullulans* је карактеристична као разлагач боја и лакова, док су врсте рода *Drechslera* познате као изазивачи плеснивости у великим депоима слика.