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EXTREMOPHILES — LINK BETWEEN EARTH AND ASTROBIOLOGY

ABSTRACT: Astrobiology studies the origin, evolution, distribution and future of life in the universe. The most promising worlds in Solar system, beyond Earth, which may harbor life are Mars and Jovian moon Europa. Extremophiles are organisms that thrive on the edge of temperature, hypersalinity, pH extremes, pressure, dryness and so on. In this paper, some extremophile cyanobacteria have been discussed as possible life forms in a scale of astrobiology. Samples were taken from solenetz and solonchak types of soil from the Vojvodina region. The main idea in this paper lies in the fact that high percentage of salt found in solonchak and solonetz gives the possibility of comparison these types of soil with "soil" on Mars, which is also rich in salt.

KEYWORDS: Astrobiology, extremophiles, cyanobacteria, halophiles

1. INTRODUCTION

1.1. *About astrobiology*

Astrobiology studies the origin, evolution, distribution and future of life in the universe. Astrobiology is multidisciplinary and brings a common biological perspective to such diverse fields as astronomy, astrophysics, biochemistry, chemistry, the ecology of extremophiles, geology, molecular biology, microbiology, paleontology, physiology, planetary sciences, space exploration and technology, without omitting law and philosophy (J a v a u x, 2006).

One of the prominent goals of astrobiology is to discover life or signs of life on planets beyond Earth. To approach this goal it will be useful to know

the physical and chemical limits for life on Earth and to understand the underlying biophysical characteristics of life that set these limits (Trent, 2000).

There are many definitions of life, but no one generally accepted. This is because it has become evident that there is such a gradual transition between abiotic structures and indisputable life forms, that any boundary drawn between them must, be based on a criterion that is questionable. Many biologists adhere to the view that the presence of DNA or at least RNA is a suitable criterion for life (Van Loon, 2005).

The most promising worlds, which may harbor living microbes are Mars and Jovian moon Europa (Seckbach and Chela-Flores, 2001). Some authors also suggest possible habitats on other icy satellites, in Venus and on the Saturnian moon Titan (Javaux, 2006).

Mars today is a frozen, dry world. The temperature of the surface ranges from –125 to +25°C (average –65°C), with wide diurnal and seasonal fluctuations. Diurnal surface temperature can span over 100 degrees (Bennett et al., 2002). The high fluctuations between midday and midnight temperatures pertain only to the several mm of the surface (Horneck, 2000). Mars has a very thin atmosphere composed of carbon dioxide (95,3%), nitrogen (2,7%), argon (1,6%) and traces of oxygen (0,15%) and water (0,03%). The average pressure on the surface of Mars is less then 1% of Earth's. Europa, a moon of Jupiter which is slightly smaller than Earth's Moon, is one of the smoothest objects in the solar system. A liquid water ocean thought to be between 50 and 100 kilometers deep surrounds its rocky interior, and is covered by a layer of water ice a few kilometers thick (Bennett et al., 2002). Following the analyses of pictures taken of the surface of Europa, it is assumed that under its heavy ice sheaths this Jovian moon contains a liquid water ocean warmed up by volcanic sources. This water body may contain living organisms similar to those found in various places on Earth (Seckbach and Chela-Flores, 2001).

Martian life may not be on the surface but in hiding in the subsurface of the dusty red Planet. The same subsurface extraterrestrial life could exist on several planets of the solar system and beyond it in the cosmos. Today the surface of Mars is inhospitable because it lacks surface liquid water, but fluids may exist in the warmer interior of the planet. Also, there is an opinion that on Mars, any liquid water would have to be a highly concentrated brine solution. Hence, that means that any present-day Martian microorganisms would be similar to terrestrial halophiles. Even if present-day life is not present on Mars, it is an interesting consideration that ancient bacteria preserved in salt deposits could be retrieved from an era when the climate of Mars was more conducive to life (Landis, 2001).

1.2. *Extremophiles and extreme environment conditions*

When biologists ask the question 'What is life?' they are constrained by the range of life forms on Earth. However, when the astrobiologist asks the same question all boundaries are removed. Imagination is the only limitation

to the astrobiologist's thinking. In case of that, extremophiles are only one of possibilities in human imagining of beginning, evolution and distribution of life in the Universe.

The special microorganisms which are able to colonize ecophysiological severe conditions are called "extremophiles". From our anthropocentric point of view these habitats are considered "extreme" although by the microorganisms themselves these places are essentials "oasis" (S e c k b a c h and C h e l a - F l o r e s, 2001).

Extremophiles thrive on the edge of temperature, hypersalinity, pH, pressure, dryness and desiccation. All three domains of life (Archea, Bacteria and Eukarya) are among the extremophiles (S e c k b a c h and C h e l a - F l o r e s, 2001). Two of these domains are prokaryotes, namely Bacteria and Archea. Extremophiles have been found in a wide range of environments on Earth, wherever there is liquid water. Extremophiles thrive at 3 km depth under the surface, in nuclear reactors, hydrothermal vents and springs, acid mine drainages and acid rivers (Rio Tinto river in Spain), in areas of high heavy metal concentrations, in halite crystals, in polar ice and lakes and in vacuums and under anaerobic conditions. They have been also found in the Dry Valleys of Antarctica and in the Atacama desert of Chili (J a v a u x, 2006).

Classification and examples of extremophiles are given in Table 1.

Tab. 1 — Classification and examples of extremophiles (G r o s s, 1998)

	Type	**Definition**	**Examples**
Temperature	hyperthermophile thermophile mesophile psychrophile	growth > 80°C growth 60—80°C 15—60°C < 15°C	*Pyrolobus fumarii*, 113°C *Synechococcus lividis* *Homo sapiens* *Psychrobacter*, some insects
Radiation			*Deinococcus radiodurans*
Pressure	barophile piezophile	Weight loving Pressure loving	Unknown For microbe, 130 MPa
Gravity	hypergravity hypogravity	> 1g < 1g	None known None known
Vacuum		tolerates vacuum (space devoid of matter)	tardigrades, insects, microbes, seeds.
Desiccation	xerophiles	anhydrobiotic	*Artemia salina*; nematodes, microbes, fungi, lichens
Salinity	halophile	Salt loving (2—5 M NaCl)	*Halobacteriacea, Dunaliella salina*
pH	alkaliphile acidophile	pH > 9 low pH loving	*Natronobacterium, Bacillus firmus* OF4, *Spirulina* spp. (all pH 10.5) *Cyanidium caldarium, Ferroplasma* sp. (both pH 0)
Oxygen tension	anaerobe microaerophil aerobe	cannot tolerate O_2 tolerates some O_2 requires O_2	*Methanococcus jannaschii* *Clostridium* *Homo sapiens*
Chemical extremes	Gases Metals	Can tolerate high concentrations of metal (metalotolerant)	*Cyanidium caldarium* (pure CO_2) *Ferroplasma acidarmanus* (Cu, As, Cd, Zn); *Ralstonia* sp. CH34 (Zn, Co, Cd, Hg, Pb)

1.2.1. Extreme Temperatures

Life on Earth is based on the chemistry of carbon in water. The temperature limits compatible with the existence of life are thus imposed by the essential properties of chemical bonds engaged in this type of chemistry at different temperatures. Two requirements are obligate. Firstly, the covalent bonds between carbon and other atoms involved in the structure of biological molecules should be sufficiently stable to allow the existence of large macromolecules with informational, catalytic properties or both. Secondly, non-covalent links (ionic and hydrogen bonds) should be labile. This is a very important point since only weak bonds can allow specific, fast and reversible interactions of biological molecules and macromolecules. These chemical limits will mainly define the upper and lower temperatures for life. And, it is known that terrestrial organisms can live in the temperature range from –12°C to 113°C (ESA, 1999).

1.2.1.1. High Temperatures

The thermophiles and hyperthermophiles are microorganisms living at high temperature. Thermophiles are found in hot waters, geothermal areas and sun-heated soils. In the hot springs of Yellowstone National Park several species, including the bacterium *Thermus aquaticus*, grow at temperatures higher than 70°C. Hyperthermophilic Archea and bacteria have been cultured from production fluids at temperature up to 110°C from oil reservoirs 3 km below the bed of North Sea (S e c k b a h, 2000). *Pyrolobus fumarii* inhabits submarine hydrothermal vents at temperature for over 100°C, and it has a highest temperature tolerance of 113°C among all forms of life (B l ö c h et al., 1997). An older publication suggested extremely high values for archaean temperature limits of cells isolated from submarine vents or "black smokers" (B a r o s s & D e m i n g, 1983). Those archean cells were reported to grow at 250°C at 265 atmospheres. It is interesting to note that the most deeply rooted organisms are thermophiles, both in Bacteria and Archea, suggesting that the earliest common ancestors might have been thermophilic microorganisms (H o r n e c k, 2000).

1.2.1.2. Low Temperatures

Life is extremely diverse in the ocean at temperatures of 2°C. Living organisms, especially microorganisms, are also present in the frozen soils of arctic and alpine environments (R u s s e l, 1992). Though, their optimal growth temperatures are ordinarily well above the temperature of the site of isolation. Those organisms with optimal growth temperatures below 15°C and minimal growth temperatures below 0°C are psychrophiles. And those capable of growth at 0°C but with optimal growth temperatures above 15°C are psychrotrophs. Psychrotrophs usually outnumber psychrophiles in a given biotope, because they can benefit more efficiently from ephemeral "warm" conditions (ESA, 1999; C a v i c c h i o l i, 2006).

1.2.2. High-Salt Environments

A group of well-studied extremophiles are the salt-loving organisms known as extreme halophiles (G a l i n s k i & T y n d a l l, 1992). Monovalent and divalent salts are essential for terrestrial life (K+, Na+, Mg++, Zn++, Fe++, Mn++) because they are required as co-catalysts in many enzymatic activities. All organisms are salt-dependent, in that sense. But, the tolerated salt concentrations are usually quite low (< 0.5%) because high salt concentrations disturb the networks of ionic interactions that shape macromolecules and hold together macromolecular complexes. The extreme halophiles, have managed to thrive in hypersaline biotopes (salines, salted lakes) up to 250—300 g/l NaCl. They are so dependent on such high salt concentrations that they cannot grow at concentrations below 10% NaCl (ESA, 1999). Halophiles may grow in hypersaline water bodies such as the Great Salt Lake (Utah) or in the Dead Sea (Israel) and in saltern evaporation ponds. Halophiles have been also observed in underground salt deposits. Many of these environments contain microorganisms that may have survived millions of years in a dormant state (S e c k b a c h, 2000; R o b e r t s, 2005).

1.2.3. Acidic and Alkaline Environments

The chemistry of life on Earth is optimised for neutral pH. Some microorganisms have been able to adapt to extreme pH conditions, from pH 0 (extremely acidic) to pH 12.5 (extremely alkaline), although maintaining their intracellular pH between pH 4 and 9 (ESA, 1999). Acidophilic microorganisms are found in all three domains of life. These organisms grow at pH levels of 0 to 4. Among the acidophiles are sulfur bacteria, Archea and phototrophic hot spring protest like the thermoacidophilic algae *Cyanidium caldarium*, *Dunaliella acidophilum* and diatoms. Hyperthermophilic archaean *Pyrolobus fumari* lives at pH range of 4 to 6,5 (B l ö c h et al., 1997; M a d i g a n & O r e n, 1999). Many bacteria and a few archaea, live at the other extreme of the pH range, from pH 9 up to pH 12, they are called the alkaliphiles (G r a n t & H o r i k o s h i, 1992). They are present everywhere on Earth. Some of them, which have been discovered in soda lakes rich in carbonates, are also halophiles (haloalkaliphiles). Most alkaliphiles are mesophiles or medium thermophilic, but there are hyperthermophilic alkaliphiles, like *Thermococcus alkaliphilus* (K e l l e r et al., 1995).

1.2.4. High-Pressure Environments

The barophilic Archaea and bacteria are present in deep subterranean locations as far as 4 km below the continental crust and on the ocean floor (pressure 1100 bar) (S e c k b a c h, 2000). The extreme pressure limit for life on Earth is unknown, environments of above 1100 bar have not been explored. Though, it might be quite high, because macromolecules and cellular consti-

tuents apparently only begin to denature at pressure of 4—5000 bar (ESA, 1999). Recent space travel to the Moon and back to Earth has shown that bacteria can tolerate long periods (at least 2.5 years) of vacuum. These observations confirm the extent of the diversity of microorganisms (S e c k b a c h, 2000).

1.3. *Extremophiles and Extraterrestrial Life*

Among early microorganisms, Cyanobacteria played a major role, inventing oxygenic photosynthesis and causing the most profound alteration in history of our planet. A few decades ago S a g a n (1961) proposed an extravagant planetary engineering plan. He suggested seeding the atmosphere of hostile planet with cyanobacteria for making these planets habitable for life. F r i e d m a n n and O c a m p o - F r i e d m a n n (1995) followed this idea of Sagan's and also proposed propagating Mars with cyanobacteria for extraterrestrial terraforming (S e c k b a c h, 2000). S v i r č e v (2005) define practical use of cyanobacteria in astrobiology, with term "astrobiotechnology".

The only criteria that is required without an exception is that every ecological niche which supports life has the presence of water in liquid form for some portion of the year. Mars is a salt-rich environment. Any present-day water would likely be a saturated salt solution, with a lower vapor pressure and a lower melt temperature than pure water (L a n d i s, 2001). Conditions for life on present-day Mars do not exist, but it may be possible that halobacteria may still be retrieved in salt deposits on Mars and cultivated in a suitable medium for growth. It is verified that bacteria on Earth can survive in salt deposits that are over 650 million years old. It is reasonable to extrapolate that possibly bacteria could survive in salt deposits for over a billion years. If this were to be true, then bacteria might be retrieved and cultured from an era dating to the time that Mars had a warm climate with liquid water, approximately 3.5 billion years ago (L a n d i s, 2001). Although the number of extreme conditions on Mars surpass all natural combinations of extreme conditions on Earth, it can be drawn a parallel between, in this case, salt rich soils on Earth and environment on Mars, which is also salt rich.

Because Mars is a salt-rich environment some halophiles might survive there. Here we are considering, basically, salinity as the reason why we should, in some cases, identify these two environments on Earth and on Mars. Vojvodina has regions that are salt rich and there are found many different strains of cyanobacteria (G a n t a r et al., 1991; S v i r č e v, 1992). In this paper are given some data of cyanobacterial strains sampled and cultivated from regions mentioned above. This data can serve for the further investigations in this field, and help us to understand better life and conditions under it can be obtained, also to understand connection between extremophiles, Earth and space.

The aim of this paper is to try in some way to connect extraterrestrial extreme environments (in this case on the Mars), with extreme environments in our region.

2. MATERIAL AND METHODS

Analysis of cyanobacterial appearance and isolation and determination of cyanobacterial strains have been performed on the samples of salt soils belonging to solonetz and solonchak types. The samples were taken from different depths (0—1 cm, 1—30 cm and 30—60 cm) during spring, summer and autumn 1996.

Solonchak soil has been found on locality near Horgoš. Solonchak belongs to halomorphic and salt-accumulatives soils. The name itself points at the type of soil which contains a lot of salt. The salt storage zone is usually the largest in surface layers, but can be found on different depths. Carbonate and bicarbonate solonchaks dominate in Vojvodina. Examined solonchaks contain 1—2% of salt, but concentration reaches up to 20% in surface rinds (Ć i r i ć, 1989). Solonetz, like solonchak, belongs to halomorphic soils but in the class of alkaline eluvial-iluvial soils. Solonetz was examined at village Kumane in Banat. If looked from outside, the soil presents mosaic made of meso-ridges and depressions. Meso-ridges are micro altitudes, 30—50 cm higher than depressions. Salt is rinsed from meso-ridges and steppe vegetation is developed. Salt is sedimented in depressions and form white layer on the surface. Usually vegetation is not developed in depressions. In a rainy season this type of soil, especially depressions, is covered by water. Because of the water proof B horizon the water cannot get through deeper layers.

Quantification of cyanobacteria has been carried out in the following way: 3g of soil were added to 50 ml of nitrogen-free (BG-11) medium (R i p k a et al., 1979) and mixed on rotary shaker for 15 min at 120 RPM. One ml of soil suspension was then filtered through a Sartorius filter (pore size 0,45 mm) and washed with 10 ml of BG-11. The filter was placed on solid BG-11 medium and incubated under cool white light at a photon fluence rate of 20 mmol m^2 s^{-1}. Dark green colonies on the filter surface were counted after 20 d and numbers of cyanobacterial colonies are expressed per g of dry soil. Individual colonies were transferred to a liquid medium (BG-11). Care was taken to select morphologically different colonies which were apparently free from bacteria and fungi. Axenic cultures were obtained by isolating individual migrating hormogonia following gamma irradiation (1 x 10^8 and 2 x 10^8 rads) (K r a u s, 1966) and antibiotic treatment (V a a r a et al., 1979). The axenic or unialgal cultures were maintained in BG-11 medium under cool-white lights at a photon fluence rate of 20 μmol m^2 s^{-1} at 24°C. Cultures of different ages were microscopically examined both in liquid and solid media.

3. RESULTS

3.1. *Number of cyanobacteria*

The amount of nitrogen-fixing cyanobacteria in the surface layer of solonchak was 39040 ind.g^{-1}, and in the surface layers of solonetz was 17533 ind.g^{-1} in absolutely dry soil in 1996 (Fig. 1). Solonetz is found with a higher

number of nitrogen-fixing cyanobacteria in depressions than on meso-ridges. This specifically refers on rainy seasons of year when depressions are filled with water. If we compare occurrence of cyanobacteria and total amount of algae in solonchak and solonetz, the percentage of cyanobacteria was 69.35% and 48.94% respectively.

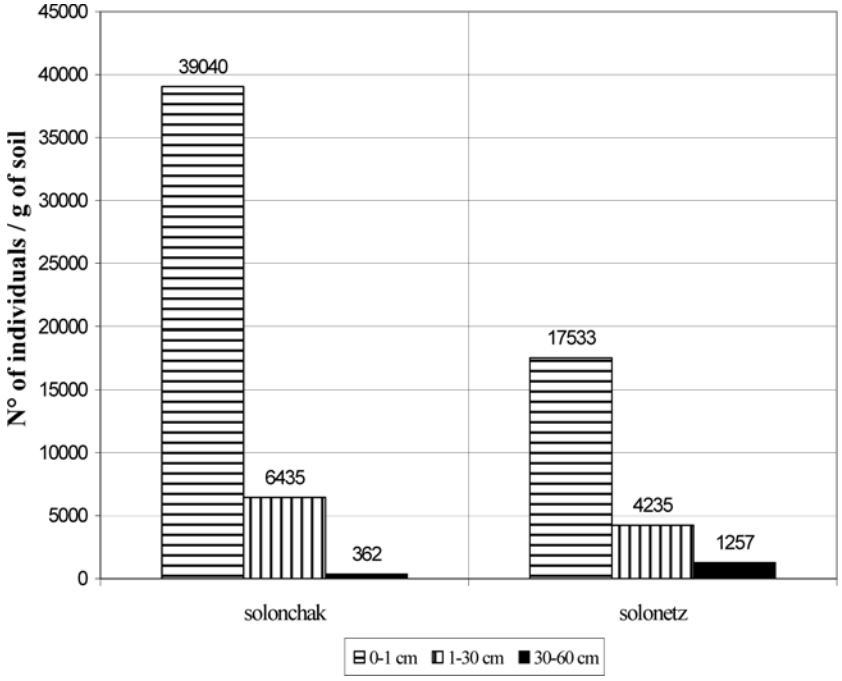

Fig. 1 — Number of nitrogen-fixing cyanobacteria in solonchak and solonetz, on different depths sampled in 1996-yearly average

3.2. *Taxonomical diversity of Cyanobacterial strains*

Fifteen strains of cyanobacteria are isolated during our investigation. Only one belongs to genus *Tolypothrix* (Fig. 2), and the rest fourteen belong to genus *Nostoc* (Fig. 3) (Tab. 1). All strains are morphologically described (stage), dimensions of vegetative cells, dimensions and percentage of heterocysts are obtained.

Tab. 1 — Morphological characteristics of isolated strains of nitrogen-fixing Cyanobacteria

Number of strain	Strain	Cyano--bacteria	Soil	Vegetative cells length x width (mm)	Heterocysts length x width (mm)	Hetero--cysts (%)	Aserial stage
1	NS AFCC 1	*Nostoc* sp.	Solonetz	3.31 x 3.31	4.78 x 3.77	8.67	+
2	NS AFCC 21	*Nostoc* sp.	Solonetz	3.03 x 3.07	5.18 x 5.10	3.06	+
3	NS AFCC 22	*Nostoc* sp.	Solonetz	3.61 x 3.35	4.93 x 4.83	14.45	+
4	NS AFCC 23	*Nostoc* sp.	Solonetz	2.55 x 2.75	4.95 x 3.82	5.98	+
5	NS AFCC 58	*Nostoc* sp.	Solonchak	2.93 x 2.55	3.43 x 2.75	6.86	+
6	NS AFCC 301	*Nostoc* sp.	Solonetz	4.96 x 4.39	5.34 x 5.04	7.16	+
7	NS AFCC 302	*Nostoc* sp.	Solonetz	3.00 x 3.79	3.79 x 3.82	6.72	+
8	NS AFCC 303	*Nostoc* sp.	Solonetz	3.91 x 3.67	3.98 x 4.30	12.40	+
9	NS AFCC 304	*Nostoc* sp.	Solonetz	3.30 x 3.48	4.34 x 4.66	8.36	+
10	NS AFCC 308	*Nostoc* sp.	Solonetz	3.34 x 3.27	3.58 x 3.62	9.57	+
11	NS AFCC 310	*Nostoc* sp.	Solonetz	3.64 x 4.18	3.94 x 4.16	6.98	+
12	NS AFCC 312	*Nostoc* sp.	Solonetz	4.02 x 4.20	4.16 x 3.86	—	+
13	NS AFCC 68	*Nostoc* sp.	Solonetz	2.55 x 3.40	3.22 x 3.52	9.09	+
14	NS AFCC 69	*Nostoc* sp.	Solonetz	2.80 x 3.82	2.93 x 4.46	10.14	+
15	NS AFCC 67	*Tolypothrix* tenuis f. terrestris	Solonetz	5.00 x 6.54	3.39 x 4.65	7.76	–

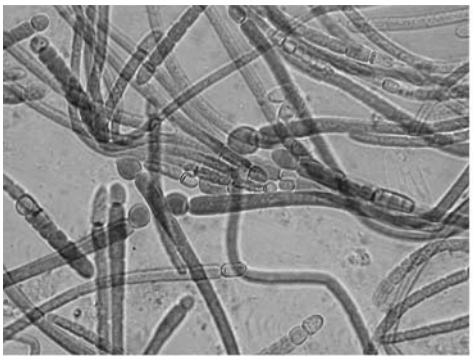

Fig. 2 — *Tolyphotrix* sp.

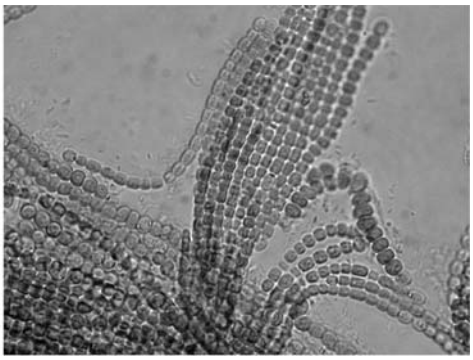

Fig. 3 — *Nostoc* sp.

4. DISCUSSION

G a n t a r et al. (1991), examined occurrence nitrogen-fixing cyanobacteria in surface layer of three types of soil in Vojvodina: black meadow, solonetz and chernozem. Results of their studies showed that higher amount of nitrogen-fixing cyanobacteria were found in solonetz, with average value in one year of 4000 ind.g^{-1}. Together with our results, this is the indicative fact that large amount of cyanobacteria is present in salt soils, and their presence is re-

gistered beneath the surface (in the range of 30—60 cm). That would be, in the case of Mars, of great significance because of the reason that intensive UV radiation is absorbed on the surface and by that its interior is sheltered from the big temperature fluctuations that are dominant at the Mars surface.

The thesis that water exists on Mars with a lower vapor pressure and a lower melt temperature than pure water goes with the facts that saturated solution of K_2CO_3, for example, will depress the freezing point of water to below 236 K. Multicomponent aqueous salt solutions can have freezing temperatures as low as 210 K and water in micron-scale pores between grains of regolith would have even lower freezing point due to capillary-pore effects (L a n d i s, 2001), which could more or less successfully bridge over, on first look, insurmountable obstacle between water in liquid state and low temperatures on Mars.

Initiated examination can serve for further, larger examinations in this directions, and also for investigations connected with isolation of extreme cyanobacteria determined genes, responsible for their "extremeness" and possibilities of forming a new species by genetic engineering which will be useful in Mars terraformation. Various microbes on Earth developed a strategy to cope with a combination of extreme conditions found in their habitats, such as the cyanobacterium *Chroococcidiopsis* which survives a large variety of extreme conditions of dryness, acidity, salt and high as well as low temperatures (W h i t t o n, 2000).

In the case that life in the Earth surroundings does not exist itself, the terraforming with Earth appropriate cyanobacterial extremophiles is still possible.

5. CONCLUSION

Despite the fact that the conditions in extreme salt-rich environments on Earth are differed than that on Mars it is reasonable to assume that from enormous diversity of strains there will be at last few of them that might serve like a good analog for possible life on Mars, prior to the cyanobacteria have been found in almost all extreme environments on Earth.

This work sets inside frames in which the link between Earth and astrobiology could be proposed, or Earth and one of life possibilities beyond its boundaries.

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ЕКСТРЕМОФИЛИ — ВЕЗА ИЗМЕЂУ ПЛАНЕТЕ ЗЕМЉЕ И АСТРОБИОЛОГИЈЕ

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Резиме

Астробиологија се бави проучавањем настанка, развића, распрострањености и будућности живота у свемиру. Места на којима је највећа вероватноћа да постоји живот ван Земље, а унутар Сунчевог система, су Марс и Јупитеров сателит Европа. Екстремофили су организми који живе и опстају у условима екстремних температура, притисака, pH вредности, екстремно сланих станишта, итд. У овом раду разматране су екстремофилне цијанобактерије као могући облици живота у астробиолошким размерама. Узорковање је обављено у Војводини, а узорци су узети са земљишта солоњец и солончак. Чињеница да солоњец и солончак садрже висок проценат соли, даје шансу поређења ова два типа земљишта са животним условима на Марсу, такође окарактерисаним високим процентом соли, што је и основна идеја овог рада.

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PECULIARITIES OF THE AUTONOMIC BALANCE ASSESSED THROUGH HEART RATE VARIABILITY ANALYSIS IN SPORTSMEN AND NONSPORTSMEN

ABSTRACT: A comparative study was used to analyze the difference in autonomic balance assessed by time and frequency domain parameters of heart rate variability (HRV) between students — athletes and non-sportsmen. Five-minute digital ECG trays were recorded in 21 students — athletes, 10 basketball players recruited from first league clubs of Novi Sad and the Serbian representatives and 11 rowers from the Novi Sad rowing club "Danubius". The control group was formed by 15 non-sportsmen, students of the Medical faculty in Novi Sad who underwent the same registrations. Time and frequency-domain of HRV were analyzed by a software developed by the company "Neurosoft", VNS-Spektr, Ivanovo, Russia. Resting heart rate in athletes was significantly lower ($p < 0.01$) than in non-sportsmen. In time-domain parameters HRV significantly higher values were present in the group of sportsmen as opposed to non-sportsmen — RRNN ($p < 0.01$), RMSSD ($p < 0.02$) and pNN50 ($p < 0.01$). In frequency-domain of HRV statistically significant difference between the two groups was observed only in normalized values of LF and HF ($p < 0.05$) and their ratio LF/HF ($p < 0.02$). LFn was larger in non-sportsmen than in students-athletes. On the other hand HFn was larger in athletes than in non-sportsmen. The LF/HF ratio was larger in non-sportsmen (2.87 ± 0.34) than in athletes (1.91 ± 0.20). After dividing the athletes recruited for this investigation into two groups (basketball players and rowers) significant level of difference ($p < 0.05$) in HRV data was present only in the VLF spectrum (2060.55 ± 290.68 ms^2 for rowers and 1303.30 ± 169.95 ms^2 for basketball players).

KEY WORDS: Heart rate variability, sportsmen

INTRODUCTION

The regular practice of physical exercise is an important factor to reduce morbidity and mortality rates of cardiovascular and all other conditions. Even though moderate exercises enhance health conditions, there are recent and con-

sistent evidences that high intensity or strenuous exercises have even more significant positive effect reducing up to two times mortality rates over a decade (Tanasescu, Leitzmann, Rimm, 2002).

Monitoring heart rate (HR) has been used to evaluate responses to different exercise stressors for a long time (Sudakov, Yumatov, Tarakanov, 1995). Recently, the attention has shifted slightly towards the field of heart rate variability (HRV). Even when HR is relatively stable, the time between two beats (R-R) can differ substantially (Achten, Jeukendrup, 2003). The variation in time between beats is being defined as HRV, and currently represents the most promising quantitative marker of autonomic activity (Task Force, 1996, Tulppo, Huikuri, 2004).

HRV is assessed by examining the beat-to-beat variations in normal R-R intervals. Originally, HRV was quantified in time-domain, i.e., R-R intervals in milliseconds (ms) plotted against time. The standard deviation of R-R intervals (SDNN), that is the square root of variance, can show short-term as well as long-term R-R interval variations. Differences between successive R-R intervals provide an index of cardiac vagal control. This can be quantified by calculating the root mean square successive difference (RMSSD) of all R-R intervals and the number of adjacent R-R intervals differing more than 50 ms expressed as a percentage of all intervals over the collection period (pNN50) (Achten, Jeukendrup, 2003).

In contrast to time-domain measures of HRV, recent developments in microprocessor technology have enabled the calculation of frequency measures based on mathematical manipulations performed on the same ECG-derived data. Instead of plotting the HRV as the change in R-R intervals over time, it is plotted as the frequency at which the length of the R-R interval changes. The main parameters on the frequency domain are very low frequency power (VLF) > 0.4 Hz, low frequency power (LF) 0.04—0.15 Hz, high frequency power (HF) 0.15—0.4 Hz, ratio between LF and HF (LF/HF) and total power (TP). HF and LF can also be expressed in normalized units, which represent the relative value of each power component in proportion to TP minus the VLF component (Achten J., Jeukendrup A. E., 2003). Some authors report even on the existence and interpretation of ultra-low frequency power (ULF) (Task Force, 1996, Tulppo, Huikuri, 2004).

Although cardiac automaticity is intrinsic to various pacemaker tissues, heart rate and rhythm are largely under the influence of the autonomic nervous system and of various hormones. The peaks at different frequencies reflect the different influences of the parasympathetic and sympathetic nervous system (Pomeranz, Macaulay, Caudill et al., 1985), involvement of some humoral mechanisms in regulation of cardiovascular functions (Kotelnikov, Nozdrachev, Odinak, 2002).

Part of the HRV is caused by respiratory sinus arrhythmia. Many studies support the view that respiratory sinus arrhythmia is generated by central coupling of the respiratory oscillator with autonomic centers in the brain stem. However a mechanical cardiopulmonary coupling has also been suggested (Mc Craty, Watkins, A., 1996, Tulppo, Huikuri, 2004). It has been shown in both clinical and experimental settings that parasympathetic

activity is a major contributor to the HF component of the HRV power spectrum (Tanasescu, Leitzmann, Rimm, 2002).

The evidence for the interpretation of the LF component is much more controversial. The LF is seen as a marker of sympathetic modulation by some authors, while others suggest it is a parameter that includes both sympathetic and parasympathetic influences. It was being ascribed to sympathetic modulation of cardiac pacemaker activity, because a variety of studies demonstrated that acute interventions that increase sympathetic nervous system activity, such as orthostatic perturbations, mental stress, handgrip exercise increase LF spectral power of the HR (Kotelnikov, Nozdrachev, Odinak, 2002).

It has been suggested that thermoregulation affects VLF heart rate variability. Studies indicate that both direct effects of temperature on pacemaker activity of the sinus node and indirect effects mediated via autonomic nervous system evoke temperature effects on HR and HRV. For not being completely attributed to any specific physiological mechanism the interpretation of VLF remains a subject of debate. The major constituent of this component is thought to be non-harmonic or fractal in nature thus assessed from short-term recordings it is a dubious measure (Mc Craty, Watkins, 1996).

The ratio of LF to HF is considered to reflect the sympatho-vagal balance (Tanasescu, Leitzmann, Rimm, 2002). This concept has been promoted by some researchers, but it lacks a physiological base. Although sympathetic and parasympathetic nervous activity constantly interacts there is no fundamental evidence that they are balanced.

The goal of our study is to reveal the differences in resting autonomic balance and in autonomic reactivity to the incremental physical load assessed by time and frequency domain parameters of heart rate variability (HRV) between students — sportsmen and non-sportsmen. In the current paper we have described only the intra-group differences in autonomic tone at the state of rest.

DESIGN OF THE STUDY AND METHODS

Subjects. All the measurements were carried out on 21 students — athletes, 10 basketball players recruited from first league clubs of Novi Sad and the Serbian representatives (age 18.7 ± 0.26 yrs, height 197 ± 2.27 cm, weight 88.9 ± 3.62 kg, active for 5.6 ± 0.4 yrs) and 11 rowers from the Novi Sad rowing club Danubius (age 16 ± 0.36 yrs, height 181.82 ± 2.38 cm, weight 72.46 ± 2.86 kg, active for 4.27 ± 0.24 yrs). The control group was formed of 15 non-sportsmen, students of the Medical faculty in Novi Sad, who had no regular physical activity (more than 3 times a week, longer than 1 hour daily) during the last six months prior the measurements. All the participants underwent a general physical examination to exclude eventual acute diseases and ailments of the cardio-respiratory and locomotor system.

Protocol. A 5-minute digital ECG was recorded in a comfortable sitting position (VNS-Spektr, Neurosoft, Ivanovo, Russia). The epoch gained from the I lead was saved in a computer for further analysis. All R-R intervals were edited by visual inspection to exclude all the undesirable beats. Time- (RRNN,

SDNN, RMSSD, pNN50) and frequency-domain analysis (after fast Fourier transformation) (TP, VLF, LF, HF, LFn, HFn, LF/HF, %VLF, %LF, %HF) was obtained after computer analysis of the digital electrophysiological signals.

The numerical data were statistically processed with the Statistica for Windows 6.0 software package. Parametric statistic was applied to calculate standard statistical factors (mean, standard deviation, error of the mean, etc.). Student t test was used to perform intra-group comparison. Statistical significance of difference was established by one-way ANOVA.

RESULTS

In this investigation we analyzed the time- and frequency-domain parameters of HRV in athletes and sedentary non-sportsmen. The results of each group are presented in table 1 (as the mean value and standard error).

Resting heart rate in athletes (68.00 ± 1.87 min^{-1}) was significantly lower ($p < 0.01$) than in nonsportsmen (79.54 ± 2.15 min^{-1}). In time-domain parameters of HRV significantly higher values were present in the group of athletes as opposed to non-sportsmen: RRNN were 896.76 ± 124.93 ms and 761.15 ± 119.93 ms respectively ($p < 0.01$), RMSSD were 48.14 ± 13.44 ms and 35.15 ± 13.78 ms respectively ($p < 0.02$) and pNN50 were 26.09 ± 2.86 and 14.17 ± 3.14 respectively ($p < 0.01$).

Tab. 1. — Resting time- and frequency-domain parameters of HRV in students — athletes and non-sportsmen (M±SE)

№	Parameters	Sportsmen	Non-sportsmen	Dif. signif.
Time-domain parameters:				
1.	Pulse (min^{-1})	68.00 ± 1.87	79.54 ± 12.15	$p < 0.01$
2.	RRNN (ms)	896.76 ± 124.93	761.15 ± 119.93	$p < 0.01$
3.	SDNN (ms)	61.10 ± 13.19	59.77 ± 14.99	$p > 0.05$
4.	RMSSD (ms)	48.14 ± 13.44	35.15 ± 13.78	$p < 0.02$
5.	pNN50 (%)	26.09 ± 12.86	14.17 ± 3,14	$p < 0.01$
6.	CV (%)	6.90 ± 10.37	7.81 ± 10.53	$p > 0.05$
Frequency-domain parameters:				
7.	TP (ms^2)	4265.67 ± 449.13	4827.92 ± 757.37	$p > 0.05$
8.	VLF (ms^2)	1699.95 ± 188.34	2275.31 ± 537.14	$p > 0.05$
9.	LF (ms^2)	1605.67 ± 222.34	1781.00 ± 254.40	$p > 0.05$
10.	HF (ms^2)	960.19 ± 152.25	771.62 ± 185.72	$p > 0.05$
11.	LFn (nu)	62.61 ± 2.37	71.08 ± 2.84	$p < 0.05$
12.	HFn (nu)	37.39 ± 2.37	28.92 ± 2.84	$p < 0.05$
13.	LF/HF	1.91 ± 0.20	2.87 ± 0.34	$p < 0.05$
14.	%VLF (%)	41.47 ± 3.07	43.12 ± 5.45	$p > 0.05$
15.	%LF (%)	36.89 ± 2.57	40.68 ± 4.45	$p > 0.05$
16.	%HF (%)	21.62 ± 1.70	16.19 ± 2.42	$p > 0.05$

Resting time- and frequency-domain parameters of HRV in students — athletes and non-sportsmen (M ± SE)

In frequency-domain of HRV statistically significant difference between the two groups was observed only in normalized values of LF and HF ($p < 0.05$) and their ratio LF/HF ($p < 0.02$). LFn was larger in non-sportsmen (71.08 ± 2.84) than in athletes (62.61 ± 2.37). On the other hand HFn was larger in athletes (37.39 ± 2.37) than in non-sportsmen (28.92 ± 2.84). The LF/HF ratio was larger in non-sportsmen (2.87±0.34) than in athletes (1.91 ± 0.20).

After dividing the athletes recruited for this investigation into two groups (basketball players and rowers) we analyzed the differences between them. As expected, basketball players were significantly taller and weigh more than rowers ($p < 0.01$). Significant level of difference ($p < 0.05$) in HRV data was present only in the VLF spectrum (2060.55 ± 290.68 ms^2 for rowers and 1303.30 ± 169.95 ms^2 for basketball players). No correlation was found among the parameters of the two sub-groups.

DISCUSSION

In light of a large number of scientific data one can not deny that aerobic exercise leads to improvement in the maximal oxygen uptake, due to at least in part, an increase of cardiac output from an increase in systolic volume (Carter, Banister, Blaber, 2003). The volume load during endurance training results in adaptive changes in many aspects of cardiovascular function. The heart improves its ability to pump blood, mainly by increasing stroke volume, which occurs because of an increase in end-diastolic volume and a small increase in left ventricular mass. In contrast, strength training results in larger increase in left ventricular mass and little or no change in ventricular volume. Endurance exercise also decreases the metabolic load on the heart at rest and at any submaximal exercise intensity — by increasing stroke volume and decreasing heart rate (Almeida, Araujo, 2003).

Long-term physical training influences cardiac rhythm. Maximal HR does not tend to change, whereas sinus bradycardia is seen in resting conditions and a slower increase in heart rate at any degree of submaximal oxygen uptake. These changes are probably related to mechanisms such as increase of venous return and systolic volume, improved myocardial contractility (Carter, Banister, Blaber, 2003). Adjustments of HR behavior from aerobic training may also be due to changes of sympathetic-vagal balance — higher parasympathetic and lower sympathetic activity — shown in many studies (Carter, Banister, Blaber, 2003).

Enhanced vagal tone to the sinus node has also been proposed to play a role in sinus bradycardia. Cardiac autonomic blockade study of humans has reported an increase in parasympathetic control of heart rate following endurance training of proper duration and intensity. Parallel to a large increase in VO_2 after endurance training, an increase in parasympathetic control of HR was shown (Pomeranz, Macaulay, Caudill, 1985). Still it remains un-

clear if the improvement of the aerobic condition from training enhances cardiac vagal tone, thus resting HR variability (Almeida, Araujo, 2003).

When looking at the time-domain variables of HRV, in most studies trained individuals had significantly higher R-R interval times, SDNN, pNN50 and RMSSD compared with their age- and weight-matched sedentary controls (Carter, Banister, Blaber). RMSDD reflects the short-term variance in HR and is the primary time-domain measure used to estimate the high--frequency beat-to-beat variations providing an estimate of the parasympathetic regulation of the heart. Increased values in athletes like in our case would indicate a parasympathetic predominance.

Whether the sympathetic nervous system also contributes to the lower resting HR is still controversial (Tanasescu, Leitzmann, Rimm, 2002). Endurance training seems to reduce the efferent sympathetic neural outflow to the sinoatrial node in the heart (Aubert, Seps, Beckers, 2003). Possible mechanisms for such a decrease in a trained individual is that the reflex heart rate response to myocardial stretch may be augmented through central, peripheral and reflex adaptations to endurance training (Tulppo, Hautala, Makikallio, 2003).

Recent studies offer a different explanation revealing intrinsic electrophysiological adaptations, such as changes in the sinus node automaticity and atrioventricular node conductivity (Smith, Hudson, Graitzer et al., 1989). Athletic training might induce intrinsic adaptations in the conduction system (mostly influencing conduction velocity), which could contribute to the higher prevalence of conductive tissue abnormalities observed in athletes (Smith, Hudson, Graitzer et al., 1989). Such indices were also present in our study. Four of the sportsmen presented VES in rest and two of them were diagnosed earlier with left branch block. A possible hypothesis as to the controversy about autonomic versus non-autonomic determinants of electrophysiological adaptations in athletes could be a fundamental difference between short- and long-term physical training programs. Short-term trainings could induce autonomic adaptations, with a reduction of sympathetic activity and an increase in parasympathetic activity (leading to bradycardia). On the other hand, long-term aerobic training, eliciting atrial and ventricular dilatation, would induce intrinsic electrophysiological adaptations and enhance parasympathetic activity (Shi, Stevens, Foresman et al., 1995).

When the data are interpreted using frequency domain variables, the results are slightly less consistent. While some investigators report TP, HF and LF (in absolute values) significantly higher in athletes compared with sedentary individuals, others reveal no such changes (Stein, Moraes, Cavalcanti et al., 2000). In our investigation changes appeared only in the normalized values of HF and LF. The HF expressed in normalized units was significantly higher in trained individuals in most of the studies. On the other hand the trained individuals in the study of E. L. Melanson and P. S. Freedson (2001) had significantly lower LF compared with their sedentary counterparts.

In our investigation athletes were characterized with lower LF/HF ratio. The ratio of LF to HF is considered to reflect the sympatho-vagal balance. Ac-

cording to this view higher values suggest a sympathetic predominance and lower parasympathetic predominance. This concept has been promoted by some researchers, but it lacks a physiological base. However it is true that both sympathetic and parasympathetic motoneurons respond to interrelated neural influences. It should therefore be noted that the LF and HF components of HRV provide a measure of the degree of autonomic fluctuation rather than a level of autonomic tone (McCraty, Watkins, 1995).

Different values of VLF power spectrum between the groups of basketball players and rowers could be explained by their metabolic and hormonal adaptations to different training regimes. Alongside temperature, endocrine factors like reproductive hormones, steroids, renin-angiotensin system also affect the VLF component of HRV (Kotelnikov, Nozdrachev, Odinak, 2002). Training regimes tend to evoke hormonal changes that will eventually result in a morpho-physiological adaptation to the presented training stimulus. Another difference between the sportsmen of these two groups is age. The rowers are significantly younger than basketball players being in the hormonally active and fluctuant period of their adolescent lives.

Longitudinal studies reveal that moderate to vigorous intensity endurance training program in adult, previously sedentary men increased markers of parasympathetic activity (significant increase in HF power) after 12 weeks (Loimaala, Huikuri, Oja et al., 2000). Others find no consistent changes in HRV, although a significant reduction of HR is observed. These authors blame the short duration of the training program and suggest that in order to obtain any effect on HRV the training program should last for a period of at least a year (Stein, Moraes, Cavalcanti et al., 2000).

Although it would be easy to conclude from the above mentioned studies that training increases HRV, studies are needed to investigate the direct effects of training on indices of HRV. While most cross-sectional studies show that endurance-trained athletes have higher HRV than their age- and weight-matched controls, the results from longitudinal studies are less conclusive. The data suggest that the duration of the exercise program might be an important factor when looking at the effects of exercise training on HRV. On the other hand vigorous training programs are necessary to induce changes in HRV, so in addition to exercise duration, exercise intensity and training volume may also play a role, yet no clear guidelines exist for the optimal training stimulus to obtain solid training adaptations (Shi, Stevens, Foresman et al., 1995).

In general, obtained data allows us to conclude that long-term (several years) adaptation to regular training programs in students — sportsmen comparing to non-sportsmen controls at the state of rest is reflected in the increase of parasympathetic activity, slight decrease in sympathetic mechanisms and in shifting of the autonomic balance to vagal predominance.

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ПОСЕБНОСТИ АУТОНОМНЕ КОНТРОЛЕ ДОБИЈЕНЕ КРОЗ АНАЛИЗУ ВАРИЈАБИЛНОСТИ СРЧАНЕ ФРЕКВЕНЦЕ КОД СПОРТИСТА И НЕСПОРТИСТА

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Резиме

Циљ истраживања била је компаративна анализа варијабилности срчане фреквенције (Heart Rate Variability — HRV) код спортиста и неспортиста. Спроведена су регистровања ЕКГ-криве у трајању од 5 минута код 21 спортисте, и то 10 прволигашких кошаркаша који играју у новосадским клубовима и репрезентативаца, односно 11 веслача из веслачког клуба "Данубиус" из Новог Сада. Контролну групу представљало је 15 неспортиста, студената Медицинског факултета у Новом Саду. Добијени временски и фреквенцијски параметри HRV-а су анализирани помоћу софтвера Неурософта, BHC-Спектр, који су развили руски научници из Иванова, Руска федерација. Вредности HRV-а у мировању су биле значајно ниже код спортиста ($p < 0.01$) у односу на вредности код неспортиста. Код анализе временских параметара вредности HRV-а су били сигнификантно више код спортиста у односу на неспортисте — RRNN ($p < 0,01$), RMSSD ($p < 0,02$) и pNN50 ($p < 0,01$). У погледу фреквенцијских параметара, статистички значајна разлика је била уочљива једино код нормираних вредности више (HFn) и ниже фреквентне области (LFn) ($p < 0,05$) и односа LF/HF ($p < 0,02$). Вредност LFn биле су више код неспортиста у односу на спортисте. С друге стране, вредности HFn биле су више код спортиста. Однос LF/HF је показивао више вредности код неспортиста (2.87 ± 0.34), него код спортиста (1.91 ± 0.20). Међусобним упоређивањем у групи спортиста, статистички значајна разлика ($p < 0.05$) код вредности HRV-а постојала је само у домену веома ниске фреквенције (VLF) (2060.55 ± 290.68 ms^2 код веслача и 1303.30 ± 169.95 ms^2 код кошаркаша).

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BODY HEIGHT AND WEIGHT AND NUTRITIONAL STATUS IN ADULT POPULATION OF NORTHWEST BAČKA AND CENTRAL BANAT (SERBIA, VOJVODINA)

ABSTRACT: Body height and weight are influenced by interaction of genetic and environmental factors but also depend upon the ethnic and socio-cultural characteristics of populations.

The aim of the study is to determine the height, weight and nutritional status of adult population of Vojvodina, as well as to establish similarities and differences among various ethnic groups, i.e. the natives of Vojvodina and newcomers from different parts of former Yugoslavia.

The investigation was conducted in 10 rural settlements of northwest Bačka and central Banat. The investigation included 608 males (mean age 41.34 ± 11.49) and 768 females (mean age 41.85 ± 10.64). Data processing included standard statistical methods, while t-test was employed for testing differences among groups. In relation to ethnic group belonging, the analysis included Serbs, Hungarians and Montenegrins, while natives and newcomers from Bosnia and Herzegovina were analysed in relation to the native land origin.

The subjects of both sexes from central Banat have greater height than the subjects from northwest Bačka. Hungarians of both sexes exhibit lower body height in comparison with all other groups, while Herzegovina newcomers have the greatest height values. For body weight, similar values are obtained in both of the areas. The average BMI in males equals 27.23 kg/m^2 in Bačka and 26.59 kg/m^2 in Banat. In females, the values are lower and equal 26.12 kg/m^2 in Bačka and 25.29 kg/m^2 in Banat.

The population of this region is characterised by great height. Natives of both sexes show markedly lower height and weight values in relation to all three newcomers groups. The greatest number of male population falls in the category of overweight (46%). Females are mostly of normal weight (47.81%), while the number of overweight and obese females equals 34.67% and 14.42%, respectively.

KEY WORDS: Body height, weight, BMI, ethnic groups, males, females, adults, Banat, Bačka, Vojvodina

INTRODUCTION

Body height and weight are the two traits most often characterised as the best indicators of physical status of populations. These traits are primarily influenced by interaction of genetic and environmental factors but also depend upon the ethnic and socio-cultural characteristics of populations. Their ratio can be used for determining body proportions and nutritional status. Today the use of BMI (body weight (kg)/body height (m^2)) is widespread in large-scale epidemiological surveys for assessing overweight, obesity and underweight (Heyward and Wagner, 2004). In the cases when BMI values exceed the standard limit set for normal weight, serious health problems can be encountered. Data from the literature have pointed out that BMI values are influenced by various factors, such as age, gender, body constitution, life-style, social status and ethnic group belonging (Roll and-Cachera et al., 1991, Shetty and James, 1994, Kuczmarski et al., 1997, Hajniš and Petrásek, 1999, Ishizaki et al., 2004, Danubio et al., 2005, Andreenko, 2005).

Due to its good geographic position, Vojvodina region has always attracted nations with different ethnic, religious, cultural, social and anthropological characteristics. According to the latest census, today in Vojvodina there are 2 031 992 inhabitants belonging to 41 ethnic groups. The largest among them are the Serbs (65%), followed by Hungarians (14.28%), Slovaks (2.79%), Montenegrins (1.75%), Rumanians (1.50%), Romanies (1.43%), Bunjevci (0.79%) etc.

According to Vlahović (1994, 2004), in present population of Vojvodina, the Serbs represent the oldest ethnic group. Hungarians were the next who settled in this region, together with Slovaks, after the liberation from Turks. In comparison with other ethnic groups, Serbs and Hungarians are the oldest groups, or so-called natives, and represent 54% of the whole population today (Serbian Statistical Annual, 2003). In addition, a large number of the inhabitants of Vojvodina (46%) are so called newcomers who arrived in this region from different parts of former Yugoslavia after the First and particularly the Second World War. In the postwar period, Montenegrins coming from all parts of their country constituted a significant percentage of newcomers in northwest Bačka. Bosnian population (Ključ, Bosanska Krupa, Bosanska Gradiška, Livno and Bosanski Petrovac) as well as Herzegovinian (Trebinje, Mostar, Ljubinje, Stolac, Konjic) mostly settled in the central Banat.

Ethnic diversity is the real treasure of Vojvodina and as such offers large possibilities for anthropological investigations. Previous investigations of the height of Montenegrins and other newcomers to Vojvodina showed that Montenegrins had greater height in comparison with natives (Gavrilović, 1960a, b) and other newcomer groups (Božić, 1976). In first investigations of Herzegovian population in Vojvodina, Gavrilović (1962) concluded that Herzegovinian adults were characterised by great height and together with Montenegrins could be classified into tallest groups on the Balkan Peninsula. In comparison with natives, they had greater weight. As for the newcomers from Bosnia (Gavrilović, 1964), they were characterised by great height, nor-

mal nutritive condition and greater weight than in natives. Following investigations of the population of Vojvodina in the area of Srem (P a v l i c a, 1996, P a v l i c a et al., 2005) showed that newcomers of both sexes had greater values of height, weight and BMI in comparison with natives.

Adults of Vojvodina were subjected to a detailed analysis in 1976, while recent studies included only adults of Srem (1996). Therefore, there is a necessity of conducting researches in other two parts of Vojvodina (Bačka and Banat).

The aim of the study is to determine the height, weight and nutritional status of adult population of Vojvodina living in rural areas in northwest Bačka and central Banat, as well as to establish similarities and differences among various ethnic groups, i.e. the natives of Vojvodina and newcomers from different parts of former Yugoslavia.

MATERIAL AND METHODS

An anthropological investigation of adult population was conducted in rural settlements of northwest Bačka and central Banat (The Province of Vojvodina, Serbia) in summer and autumn 2004 and in autumn 2005. It was a cross-sectional survey conducted in compliance with recommendations of In-

Fig. 1 — Map of North Serbia — Vojvodina

ternational Biological Program (IBP) and World Health Organisation (WHO) and original Martin's anthropometric instrument was employed (Sieber Hegner, Switzerland).

For all of the subjects height and weight were measured and BMI was obtained. Subsequently, the subjects were classified into categories that complied with the criteria of WHO (2000).

The investigation included 279 males (mean age 43.35 ± 10.85) and 367 females (mean age 42.58 ± 10.73) living in northwest Bačka (Mali Iđoš, Lovćenac, Sivac and Crvenka) and 329 (mean age 39.33 ± 12.84) males and 401 females (mean age 41.13 ± 10.56) living in central Banat (Žitište, Klek, Novi Bečej, Ravni Topolovac, Sečanj and Srpska Crnja) (Figure 1).

The data obtained from all subjects in this investigation included the date and place of birth, ethnic group and native land origin. Decimal age obtained from the date of investigation and the date of birth was calculated for all of the subjects. As for data regarding native land origin, they included the place of birth of grandparents. The subjects whose grandparents were born in Vojvodina are classified as natives, while those whose grandparents were born in other parts of former Yugoslavia (Montenegro, Bosnia, Herzegovina, Lika, Kosovo, Dalmatia, Croatia) are classified as newcomers. Those subjects whose grandparents from one side are natives and from the other newcomers are classified as "mixed population".

The data were processed in relation to the sex, ethnic group and native land origin. Data processing included standard statistical methods, while t-test for large sample was employed for testing differences among groups. In relation to ethnic group belonging, the analysis included Serbs, Hungarians and Montenegrins, while natives and newcomers from Bosnia and Herzegovina were analysed in relation to the native land origin. Newcomers from other parts of former Yugoslavia and "mixed population" were not analysed separately, since their number was insufficient for a valid analysis.

RESULTS

The distribution of northwest Bačka subjects of both sexes with reference to their ethnic group belonging and native land origin is shown in Table 1.

Tab. 1 — Distribution of the subjects by ethnic group and native land origin in northwest Bačka settlements

Investigation site	Natives		Hungarians		Newcomers from Montenegro		Newcomers from Bosnia		Newcomers from different parts of former Yugoslavia		Mixed population	
	n	%	n	%	n	%	n	%	n	%	n	%
Lovćenac	11	10.48	—	—	65	61.90	13	12.38	13	12.38	3	2.86
Sivac	60	31.09	5	2.59	93	48.19	4	2.07	19	9.84	12	6.21
Crvenka	25	22.52	—	—	21	18.92	32	29.73	30	27.03	2	1.81
Mali Iđoš	3	0.84	227	95.78	—	—	—	—	6	2.53	2	0.84
Total	99	15,33	232	35,91	179	27,71	49	7,59	68	10,53	19	2,94

It includes 4 settlements and the total population is divided into 6 categories. The group of natives includes the population of Serbian origin and other smaller ethnic groups whose grandparents from both sides were born in Vojvodina. The highest percentage is observed in the following 3 categories: Hungarians (35.91%), newcomers from Montenegro (27.71%) and natives (15.33%). As for other categories, they are present in lower percentage.

The distribution of central Banat subjects in relation to the native land origin (Table 2) shows that natives are the most numerous group (33.70%), followed by newcomers from Bosnia (25.89%) and Herzegovina (21.51%). The other categories are present in lower percentage. The distribution of northwest Bačka and central Banat subjects obtained in this study complies with the percentage data obtained in the latest census in 2002.

Tab. 2 — Distribution of the subjects by native land origin in central Banat settlements

Investigation site	Natives		Newcomers from Bosnia		Newcomers from Herzegovina		Newcomers from different parts of former Yugoslavia		Mixed population	
	n	%	n	%	n	%	n	%	n	%
Žitište	37	25.52	55	39.31	21	14.48	20	13.79	10	6.89
Klek	31	14.69	61	28.91	71	33.65	36	17.06	12	5.68
Novi Bečej	110	78.57	11	7.86	1	0.71	12	8.57	6	4.28
Ravni Topolovac	3	6.45	13	41.93	14	45.16	1	3.22	1	3.22
Sečanj	27	25.71	18	17.14	43	40.95	9	8.57	7	15.23
Sr. Crnja	38	38.00	31	31.00	7	7.14	14	14.28	10	10.20
Total	246	33,70	189	25,89	157	21,51	92	12,60	46	6,30

The basic parameters of descriptive statistical analysis and t-test values for height, weight and BMI of the total number of male and female subjects are shown in Tables 3 and 4, respectively.

Tab. 3 — Distribution of body height, weight and BMI in northwest Bačka and central Banat — males

	Northwest Bačka				Central Banat			
	Age	Body height (cm)	Body weight (kg)	BMI (kg/m^2)	Age	Body height (cm)	Body weight (kg)	BMI (kg/m^2)
n	279	279	279	279	329	329	329	329
Mean	43.35	174.78	83.32	27.23	39.33	178.40	84.72	26.59
SD	10.85	7.23	14.44	4.16	12.14	7.24	13.00	3.56
Min	18.01	156.50	51.50	18.02	17.87	157.50	55.00	18.38
Max	68.53	199.00	138.00	40.76	66.15	202.00	125.00	37.03
CV	25.02	4.14	17.33	15.28	30.87	4.06	15.35	13.38
t-test				2.01*		6.15**	1.33	

* $p < 0.05$; ** $p < 0.01$

It is observed in Table 3 that the average age is 43.35 years for northwest Bačka subjects and 39.33 years for the subjects of central Banat. The males

from central Banat show greater height (178.40 cm) than the males from northwest Backa (174.78 cm), this difference being statistically significant ($p < 0.01$). Coefficient of variation for both of the groups equals approximately 4%, thus indicating homogeneity of the samples.

The average weight in males of central Banat and northwest Bačka equals 84.72 kg and 83.32 kg, respectively; this difference is statistically insignificant. This trait, however, shows greater coefficient of variation (15%), which complies with usual manifestation of this trait in a human population. The average BMI in both groups of the subjects is approximately 27 kg/m^2.

Tab. 4 — Distribution of body height, weight and BMI in northwest Bačka and central Banat — females

	Northwest Bačka				Central Banat			
	Age	Body height (cm)	Body weight (kg)	BMI (kg/m^2)	Age	Body height (cm)	Body weight (kg)	BMI (kg/m^2)
n	367	367	367	367	401	401	401	401
Mean	42.58	162.38	68.79	26.12	41.13	163.06	67.21	25.29
SD	10.73	6.36	12.36	4.63	10.56	6.32	11.34	4.25
Min	15.94	140.00	40.00	16.73	19.29	143.00	45.00	17.06
Max	69.52	179.00	122.00	42.08	67.33	183.20	115.00	43.28
CV	25.19	3.91	17.96	17.72	25.67	3.87	16.88	16.81
t-test			1.84	2.58**		1.49		

* $p < 0.05$; ** $p < 0.01$

As for females, both of the groups (Table 4) are above 40 years of age. Alike males, greater height is observed in central Banat females (163.06 cm) than in females from northwest Bačka (162.38 cm), but this difference shows no statistical significance. Variation coefficients indicate the homogeneity of the female sample.

The average weight of females from Bačka is 1.5 kg greater than the average weight of Banat females, this making no significant statistical difference. As for the average BMI, it is lower than in males.

Tab. 5 — Distribution of body height, weight and BMI in natives and newcomers from different parts of former Yugoslavia — males

Characteristics		Serbs natives in Bačka	Serbs natives in Banat	Hungarians	Newcomers from Montenegro	Newcomers from Herzegovina	Newcomers from Bosnia
Body height	n	44	63	104	82	77	87
	Mean	175.8	176.50	172.42	176.33	180.74	179.48
	SD	6.56	7.00	7.00	7.18	7.07	5.83
Body weight	Mean	82.8	83.10	80.86	86.74	88.52	86.22
	SD	13.63	14.26	14.74	13.81	13.02	11.69
BMI	Mean	26.7	26.60	27.14	27.89	27.10	26.80
	SD	3.32	3.90	4.37	3.99	3.70	3.40

Tables 5 and 6 show the distribution of height, weight and BMI in natives and newcomers. In both sexes there are 6 categories representing largest ethnic and native land groups. It can be observed in Table 5 that the lowest height is recorded in Hungarians (172.42 cm), and the greatest in newcomers from Herzegovina (180.74 cm) and Bosnia (179.48 cm). These differences are statistically significant ($p < 0.01$). Hungarions show significantly lower values of height in comparison with both groups of Serb natives and Montenegrins ($p < 0.01$). Approximately equal values of height are observed in natives of Serbian nationality and newcomers from Montenegro.

A similar distribution is observed with reference to weight. The lowest values are recorded in Hungarians (80.86 kg) and the highest in newcomers from Herzegovina (88.52 kg) and Bosnia (86.22 kg), this being statistically significant ($p < 0.01$). It can be observed that natives of Serbian and Hungarian nationality are characterised by significantly lower weight in relation to the three groups of newcomers ($p < 0.01$).

The average BMI values are mostly equal. The lowest BMI is recorded in Serbian natives in Banat (26.60 kg/m^2) and the highest in Montenegrin newcomers (27.89 kg/m^2), making this difference statistically significant ($p < 0.05$). No statistically differences are observed in other categories.

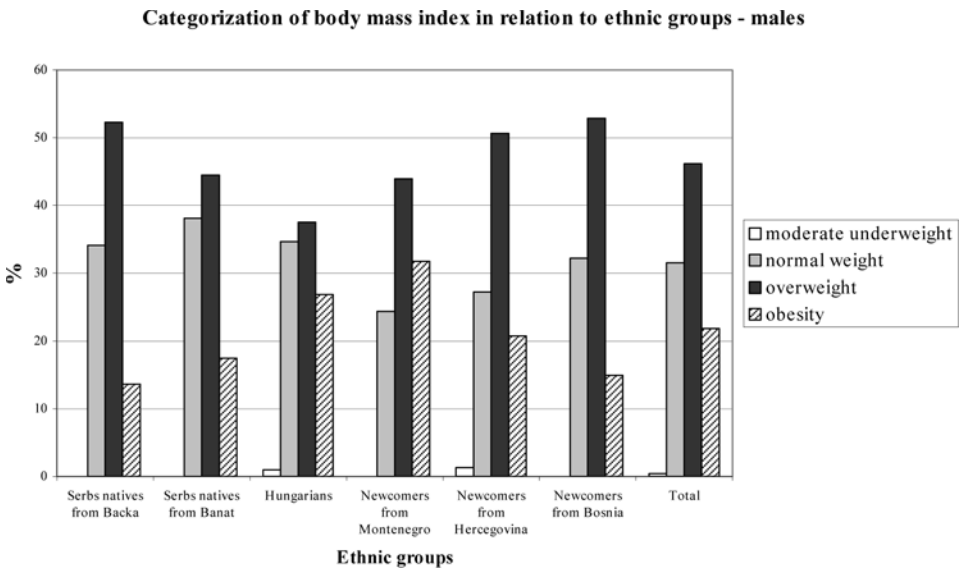
Tab. 6 — Distribution of body height, weight and BMI in natives and newcomers from different parts of former Yugoslavia — females

Characteristics		Serbs natives in Bačka	Serbs natives in Banat	Hungarians	Newcomers from Montenegro	Newcomers from Herzegovina	Newcomers from Bosnia
Body height	n	34	90	145	95	80	104
	Mean	163.5	162.33	160.63	164.58	166.48	163.24
	SD	6.02	6.04	6.39	5.97	6.14	5.78
Body weight	Mean	71.5	66.48	67.67	69.18	68.41	67.22
	SD	13.22	11.50	12.63	12.66	10.67	11.04
BMI	Mean	26.9	25.22	26.23	25.62	24.74	22.58
	SD	5.49	4.10	4.62	4.83	3.91	4.24

In females (Table 6), lower height is observed in Serbian natives in Banat and Hungarians, while newcomers from Montenegro and Herzegovina show greater height values. Significantly lower height is observed in Hungarian females (160.63 cm) in comparison with all native and newcomer groups ($p < 0.05$ and $p < 0.01$, respectively). The greatest height is recorded in female newcomers from Herzegovina (166.48 cm).

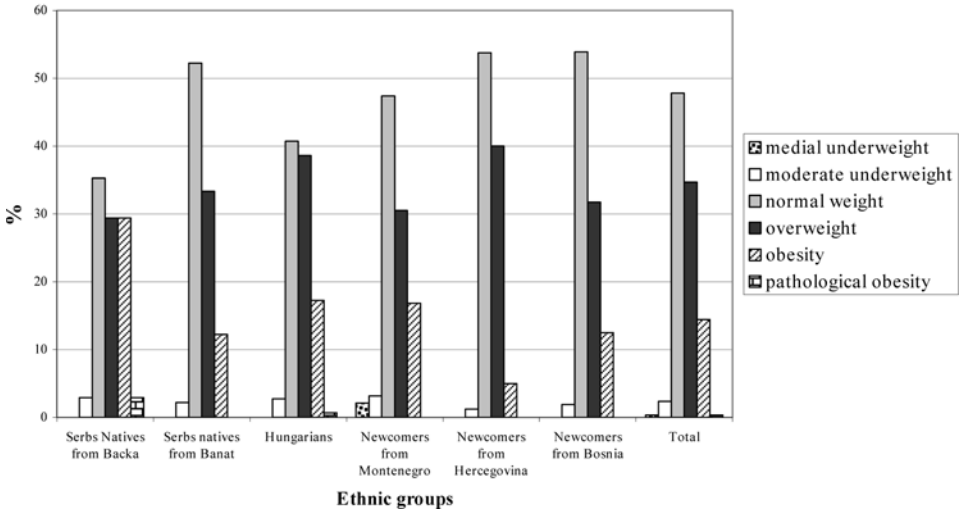
The lowest weight value is observed in Serbian females from Banat (66.48 kg) and the heighest in Serbian females from Bačka (71.5 kg). Differences observed among individual groups are statistically insignificant.

The average BMI shows greater variability than in males ranging from 22.58 kg/m^2 (female newcomers from Bosnia) to 26.9 kg/m^2 (Serbian natives in Bačka). Female newcomers from Bosnia show significantly lower BMI ($p < 0.01$) in comparison with other groups.



Graph. 1 — Categorization of body mass index in relation to ethnic groups — males

Graph 1 and 2 present the category of BMI in both sexes of different ethnic groups. In males (Graph 1), the identical trend of BMI distribution in all groups is observed. The greatest number of subjects are overweight (25—29.9 kg/m^2). In general, 46% of subjects from all ethnic groups are overweight. Within this category, the smallest number is observed in Hungarians



Graph. 2 — Categorization of body mass index in relation to ethnic groups — females

(37.50%) and the greatest in newcomers from Bosnia (52.87%) and natives from Bačka (52.27%). The percentage of subjects with normal weight equals 31.51%. This refers to all ethnic groups with an exception of newcomers from Montenegro and Herzegovina where this percentage is lower. Obesity (BMI 30—39.9 kg/m^2) is present in 21.88%, varying from 13.64% (Serbian natives in Bačka) to 31.71% (newcomers from Montenegro). In none of the cases pathological obesity has been observed.

In females (Graph. 2), the identical trend of BMI distribution for all ethnic groups is also observed. Female subjects are mostly of normal weight (47.81%), while a smaller number falls into the category of overweight and obese (34.67% and 14.42% respectively). The same percentage (0.36%) is recorded with reference to pathological obesity and medial underweight.

DISCUSSION

The paper analyses height, weight and nutritive status in adult population of northwest Bačka and central Banat (Serbia). The analysis included 1376 subjects of both sexes in 10 rural settlements. The subjects' average age is above 40 years, except males from central Banat (39 years). Apart from natives who are mostly of Serbian and Hungarian nationality, the analysis included the offspring of newcomers of Serbian nationality that settled in this region. They mostly arrived from Bosnia and Herzegovina in the period from 1946. to 1952.

The results obtained in the analysis of the whole sample in northwest Bačka and central Banat indicate that the population of this region is characterised by great height. The subjects of both sexes from central Banat have greater height than the subjects from northwest Bačka. This is particularly observed in males, where the difference is statistically significant (3.62 cm), which is not the case with females (0.68 cm). The reason for such distribution probably lies in the fact that most of the population of northwest Bačka are natives, opposed to central Banat where there are a lot of newcomers from Herzegovina and Bosnia. This population, as previous studies have shown (Gavrilović, 1962, 1964, Božić, 1976), is characterised by greater height than it is the case with natives. In relation to some studies of the Czech and Slovak populations of the same age (Hajniš and Petrásek, 1999), similar values of body height are observed.

The analysis based upon ethnic group belonging and native land origin shows that Hungarians of both sexes exhibit lower body height in comparison with all other groups, while Herzegovina newcomers have the greatest height values. Natives of both sexes show markedly lower height values in relation to all three newcomers groups, which is in compliance with earlier investigations of body height of natives and newcomers (Gavrilović, 1960a, b, 1962, 1964, Božić, 1976, Pavlica, 1996, Pavlica et al., 2005).

With reference to the first investigations of Herzegovinian (Gavrilović, 1962) and Bosnian (Gavrilović, 1964) populations in Vojvodina, which included only male subjects, an increase in height is observed (5.74 cm

in Herzegovinians and 6.48 cm in Bosnians). It points to the acceleration, i.e., higher growth and physical development, a phenomenon which has been present worldwide.

As for body weight, similar values are obtained in both of the areas included in this investigation. In males of central Banat and northwest Bačka body weight equals 84.72 kg and 83.32 kg, respectively. Speaking of females, the average weight is greater in Bačka (68.79 kg) than in Banat (67.21 kg), but in neither of the cases these differences are statistically significant. Similar values of body weight are observed in the Czech and Slovak populations (Hajniš and Petrásek 1999).

The analysis of the results by ethnic group belonging and native land origin indicates the same distribution of males' weight as it is the case with height. The lowest body weight is recorded in Hungarian males (80.86 kg) and the highest in newcomers from Herzegovina (88.52 kg) and Bosnia (86.22 kg). Natives show lower weight values as compared with newcomers, which complies with previous investigations of body weight in Vojvodina (Gavrilović, 1962, 1964, Pavlica, 1996, Pavlica et al., 2005). Opposed to males, greater uniformity is observed in females' weight, which is also in compliance with previously obtained results (Pavlica, 1996). The lowest body weight is recorded in Serbian females from Banat (66.48 kg) and the highest in Serbian females from Bačka (71.5 kg). Differences observed among various groups are not statistically significant. In comparison with previous studies (Gavrilović, 1962, 1964) there has been an increase in body weight equalling approximately 12 kg.

In the sample of the total population of Vojvodina the average BMI in males equals 27.23 kg/m^2 in Bačka and 26.59 kg/m^2 in Banat. In females, the values are lower and equal 26.12 kg/m^2 in Bačka and 25.29 kg/m^2 in Banat. These values are markedly higher in comparison with the results obtained for the same age in Japan (Ishizaki et al., 2004), in Italian immigrants (Danubio et al., 2005), in France (Rolland-Cachera et al., 1991) and in developing countries (Shetty and James, 1994). American population measured in the period between 1988 and 1994 (Kuczmarski et al., 1997) shows similar averages for 30—59 year-old subjects (males 27.1, females 27.0). The same is observed in the Czech and Slovak population (Hajniš and Petrásek, 1999).

The average BMI of different ethnic and native land origin groups is almost identical in males and indicates the condition of overweight in all of the categories. Significant differences are only observed between the lowest and the highest values (Serbian natives and Montenegrin newcomers).

With regard to females, the average BMI is lower but varies in a larger span. The lowest BMI is recorded in female newcomers from Bosnia, this being a significant difference when compared to all other groups.

In BMI categorisation of different ethnic groups the same trend of distribution is observed. The greatest number of male population falls in the category of overweight. In all ethnic groups the percentage of individuals with BMI ranging 25—29.9 kg/m^2 equals 46%, with the largest number recorded in Bosnian newcomers and the smallest in Hungarians. The number of indivi-

duals of normal weight is above 30%. The percentage of obese males varies from 13.64% (Serbian natives in Bačka) to 31.71% (Montenegrin newcomers). The first degree obesity is recorded in all of the subjects (30.0 kg/m^2 — 34.9 kg/m^2). No cases of pathological obesity are recorded. A similar distribution of BMI categories is observed in male Bulgarians (Andreenko, 2005).

Females of different ethnic groups are mostly of normal weight (47.81%), while the number of overweight and obese females equals 34.67% and 14.42%, respectively. Pathological obesity and moderate underweight is present in 0.36%. In relation to the results of BMI studies from different parts of the world (Shetty and James, 1994), our results point to certain similarities between the population of Vojvodina and American and Hungarian populations, as far as the percentage of the overweight and obese is concerned. In comparison with developing countries of South America and Africa, however, the percentage of the overweight and obese is markedly higher in Vojvodina region.

Adults of this region is characterised by great height. Natives of both sexes show markedly lower height and weight values in relation to newcomers groups. The greatest number of male population falls in the category of overweight (46%). Females are mostly of normal weight (47.81%), while the number of overweight and obese females equals 34.67% and 14.42%, respectively.

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ВИСИНА ТЕЛА, МАСА ТЕЛА И СТАЊЕ УХРАЊЕНОСТИ КОД ОДРАСЛОГ СТАНОВНИШТВА СЕВЕРОЗАПАДНЕ БАЧКЕ И ЦЕНТРАЛНОГ БАНАТА (СРБИЈА, ВОЈВОДИНА)

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Резиме

Висина и тежина тела су под утицајем генетских и спољашњих чинилаца, али на њихово формирање утичу и етничке и социокултурне особине популација.

Циљ рада је био да се утврде висина, тежина и стање ухрањености одраслих становника Војводине, као и сличности и разлике између етничких група, староседелаца Војводине и досељеника из различитих крајева бивше Југославије.

Истраживање је спроведено у 10 руралних насеља у северозападној Бачкој и централном Банату. Истраживањем је обухваћено 608 мушкараца (41.34 ± 11.49) и 768 жена (41.85 ± 10.64). При обради података коришћена је стандардна статистичка метода, а разлике између група су тестиране т-тестом. Према етничкој припадности анализирани су Срби, Мађари и Црногорци, а према завичајном пореклу староседеоци и досељеници из Босне и Херцеговине.

Испитаници оба пола из централног Баната имали су веће вредности висине тела од испитаника из северозападне Бачке. Мађари оба пола имали су мању висину тела у поређењу са свим осталим групама, док су досељеници из Херцеговине имали највећу телесну висину. За масу тела уочене су сличне вредности у оба испитана региона. Просечна вредност БМИ код мушкараца је 27.23 kg/m^2 у Бачкој и 26.59 kg/m^2 у Банату. Код жена вредности су ниже и износе 26.12 kg/m^2 у Бачкој и 25.29 kg/m^2 у Банату.

Популација ових региона карактерише се високим стасом. Староседеоци оба пола имају значајно ниже вредности висине и тежине тела од све три групе досељеника. Мушкарци су претежно прекомерно тешки (46%). Жене су већином нормално ухрањене (47.81%), прекомерно тешких је 34.67%, а гојазних 14.42%.

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PHYSIOLOGICAL AND GENETIC BASIS OF PLANT TOLERANCE TO EXCESS BORON

ABSTRACT: Boron (B) deficit as well as excess may significantly limit the organic production in plants. In extreme cases they may kill the affected plants. Boron excess occurs primarily in arid and semiarid regions, in saline soils or in consequence to human action. Excessive boron concentrations retard plant growth and cause physiological and morphological changes (chlorosis and necrosis) first of all in leaf tips and then in marginal or intercostal parts of the lamina. Physiological mechanisms of plant tolerance to boron excess have not been studied in sufficient detail. The predominant opinion holds that they are based on restricted uptake and accumulation of boron in the root and aboveground plant parts. Significant differences in boron excess tolerance have been observed not only between different crops but even between different genotypes of the same crop. This has enabled the breeding of crop genotypes and crops adapted to growing on soils rich in available boron and intensified the research on the inheritance of plant tolerance to high B concentration. Sources of tolerance to high B concentration have been found in many crops (wheat, mustard, pea, lentil, eucalypt). Using different molecular techniques based on PCR (RAPD, SRAP), plant parents and progenies have been analyzed in an attempt to map as precisely as possible the position of B-tolerant genes. Small grains have been studied in greatest detail for inheritance of B tolerance. B tolerance in wheat is controlled by at least four additive genes, *Bo1, Bo2, Bo3* and *Bo4*. Consequently, there exists a broad range of tolerance levels. Studies of *Arabidopsis* have broadened our understanding of regulation mechanisms of B transport from roots to above ground parts, allowing more direct genetic manipulations.

KEY WORDS: boron toxicity, mechanism of action, inheritance of tolerance, crop plants

INTRODUCTION

Effect of a chemical element on vital processes and organic production in plants depends primarily on its physiological role in plant growth and development and its concentration in the environment. Optimum provision with essential elements is a prerequisite for normal growth and development of plants. If an element's concentration in nutritive medium is above the optimum but below a toxic level, it may accumulate in plant tissues, i.e., there occurs a latent excess of that element. If the element's concentration in nutritive medium

exceeds the toxic level, disturbances in plant vital processes may affect plant condition and chemical composition, organic matter production and they may cause death of the affected plants.

Boron is a biogenous element for higher plant. Boron excess as well as shortage cause physiological and morphological changes in plants. The range between boron deficit and excess is quite narrow. Excess B in nutritive medium is a frequent limiting factor in crop production. Reasons for the occurrence of excess B may be lithogenic and pedogenetic processes or human activities. Symptoms of B toxic action on crop plants were observed and identified for the first time in the early 1930s. Signs of B excess have been registered in southern Australia, western Asia, northern Africa, Turkey (central Anatolia) and other parts of the world. Previously, scientific attention has been focused on B shortage. Presently, the attention is beginning to shift towards B excess, as the practical aspect of this problem gains importance.

SOURCES OF BORON

Boron is widely distributed in nature, but in exceedingly low concentrations. Its content in Earth's crust is approximately 0.0003%. In nature it is exclusively bound to oxygen, most frequently in the form of polyborates such as borax, kernite or colemanite. Turmaline is the most important B-containing mineral (3 to 4%). Total B content in soil ranges from 20 to 200 mg kg^{-1}, most frequently from 30 to 40 mg kg^{-1}. A major portion of soil B is not available to plants. Of total B in soil solution, only 5 to 15% are available to plants, mostly in the form of boric acid (Gupta, 1979). B concentration in soil solution typically ranges from 0.01 to 5 mg L^{-1} (Schilling, 2000). Soils in moderate climate regions also contain available B in the form of calcium borate. In arid regions, available Ca-, Na- and K-borate can be found in increased amounts. Soils developed from igneous rocks are typically poorer in B than those developed from sedimentary rocks. Average B proportion in arid and semiarid soils is higher than in humid soils. This is why B excess is more frequent in semiarid and arid regions than in humid ones. Because of facilitated B leaching in humid regions, their soils are typically poorer in B, especially sandy soils. Soils in littoral zones frequently have an increased B content because surf spray contains 4 to 5 mg B L^{-1}. In arid conditions, especially in soils subject to sodium accumulation, Na- and Ca-borate accumulate on soil surface. Besides, irrigation water in arid regions frequently has an increased B content. Also, soils developed from marine sediments have an increased B content. Toxic concentrations of B had been found in water extracts of most saline soils from the Vojvodina Province (Miljković, 1968).

B availability to plants depends on a large number of factors (Kastori 1990). Soil organic matter has a particularly positive influence on B availability. The amount of available B in soil varies in the course of the year. B availability decreases in proportion to increase in pH, except in the case of saline soils. If a high pH is due to a high Na concentration, B availability to plants tends to increase.

Human activities may affect B content in soil. The problem of B excess intensifies in greenhouses used for production of vegetables and decorative plants. Use of irrigation water containing 0.5 to 1.0 mg^{-1} of B may gradually lead to its excess (Nable and Paull, 1991). Plant species vary in reaction to B content in irrigation water. According to Nable et al. (1997), B content in irrigation water should range from 0.3 do 1 mg^{-1}, 1 to 2.1 mg^{-1} and 2.1 to 4.0 mg^{-1} for susceptible, medium tolerant and tolerant species, respectively. Irrigation with tap water containing an increased B content also may cause accumulation of excess B in plants. Tap water with B content of 2 mg^{-1} or more is considered as unsuitable for irrigation. Ground waters also may have a high B content. Fertilization with urban compost (Purves and Mackenzie, 1974) or residues containing a large proportion of brown coal may lead to B excess because of a relatively high B content in brown coal. There was a case when toxic B action occurred after the application of manure that had been treated with boric acid to control insects and their larvae. Excess B may also occur in clay and enameled pots (Mitscherlich pots) used for laboratory growth trials. Barren soil with high B content removed from strip mines and used as landfill also may cause B toxicity in plants. Industrial and urban air pollutions, especially if they contain large amounts of ash, may contribute to B accumulation in soils and plants (Kozma and Tölgyesi, 1978). According to Romneg et al. (1977), risk of B accumulation in soils and plants is present in the vicinity of coal-fueled power plants because lignite contains up to 300 mg kg^{-1} B, and its ash, which is an air pollutant, contains from 19 to 51 mg kg^{-1} B. Conifers are particularly sensitive to air pollution. B accumulation in soil may result from systematic application of B fertilizers of B-enriched mineral fertilizers. According to Reisenauer et al. (1973) toxic B action may be expected to occur in field crops when B content extracted from soil with boiling water exceeds 5 mg kg^{-1}, and B shortage when B content goes below 1 mg kg^{-1}. Symptoms of B excess in sandy soil, loamy sand, loamy sand and loamy and clayey soils occur when B content extracted with boiling water exceeds 0.80 mg kg^{-1}, 1.00 mg kg^{-1}, 1.20 mg kg^{-1} and 2.00 mg kg^{-1} (Robertson et al., 1975). Considering the narrow range between optimum and toxic B concentrations, it is necessary to be careful when applying B fertilizers, especially to light soils.

PHYSIOLOGICAL BASIS OF TOLERANCE

Boron movement in plants is mostly associated with transpiration course, which explains why it accumulates in leaf tips and margins. According to Petrović and Kastori (1983), B concentration in leaves identified for excess B fell from 212 mg B kg^{-1} DM at leaf margin to 45 mg B kg^{-1} DM in the center of the leaf. Because of such distribution pattern, first signs of B excess occur on leaf tips and margins, in the form of a necrosis, first in mature and later in juvenile leaves. In the case of high B excess, symptoms also occur in intercostal parts of the lamina, first as brown spots which necrotize later on. Progression of symptoms of B excess depends also on B concentration in the

environment. If B accumulation in plants is gradual, chlorotic spots first occur on leaves which then become necrotic. If B accumulation is rapid, necrotic spots develop immediately. Also, various plant organs react differently to high B concentration. After Kluge (1990) symptoms of B excess on wheat leaves appear at lower B concentration than the reduction of grain yield. B content in wheat seeds may increase 20 times without negatively affecting germination rate and seedling growth (Nable and Paull, 1990). In addition to leaf necrosis, excess B causes other morphological and physiological changes such as reduced plant height, impaired growth of aboveground plant parts (Paull et al., 1990) and roots (Huang and Graham, 1990).

Some plant species exhibit specific symptoms of B excess (Bergmann and Neubert, 1976). In wheat, the stem acquires a pink-red color. In tomato, there occurs the curling of top leaves. In grapevine, the edges of young leaves curl dorsally and pollen variability is reduced. In rice, numbers of spikes and grains per spike are reduced. Special care should be exercised when applying B in orchards. Apple fruits mature earlier and such fruits are susceptible to various physiological disorders. If B content in apple fruits is above 40 mg kg^{-1} DM, the fruits have shorter shelf life.

Morphological changes of plants are not a reliable indicator of B excess since toxic concentrations of other elements may cause similar symptoms. To reliably establish B status in plants, it is necessary to determine first B concentration in plants and then in the nutritive substrate. Here it should be kept in mind that the total B content in soil does not provide a reliable information of B availability to plants.

The investigations conducted so far seem to suggest that plant tolerance to toxic concentrations of B is based on several mechanisms. Nable (1988) stated that sensitivity to toxic B concentrations in wheat cultivars depends primarily on tissue capacity to release B. Paull et al. (1992) pointed out the importance of B release via roots, slow B uptake and limited B translocation to the aboveground parts, singling out the intensity of B uptake as the most important factor. According to Chantachume et al. (1995) wheat genotypes tolerant to B excess have a longer root system than sensitive genotypes. Root length could thus be used as a criterion in breeding for plant tolerance to excess B. In barley, reduced B uptake is based on genetically controlled slow passive B transport through the plasmatic membrane of root cells, and not on the anatomy of the root or transpiration intensity (Nable and Paull, 1991). As sugars form complexes with B, high sugar concentration may reduce B toxicity (Yokota and Konishi, 1990). While some authors claim that plant species capable of effective B bonding in the cell wall have improved tolerance to high B concentrations, others hold the opinion that B bonding in the cell wall does not play an important role in B detoxication (Dannel et al., 1998). It is the prevailing opinion that the mechanism of plant tolerance to high B concentrations is based on a restricted uptake of B and thus on its lower accumulation in roots and aboveground parts.

A high B concentration in the cytosol causes disturbances in plant metabolism, also manifested through B complexing with NAD^+ or rRNA or through a specific inhibition of ureide metabolism (Lukaszewski et al., 1992). Si-

milarities exist between mechanisms of plant tolerance to high concentrations of salt and high concentrations of B (Nable et al., 1997).

Unfavorable effects of B excess in soil can be mitigated to a certain measure by calcium application, use of irrigation water low in B or the application of 90 do 100 kg N ha^{-1}, in the form of lime-ammonium saltpeter. It was observed that satisfactory soil provision with K may lessen the harmful effects of excess B (Shalaby and Kádár, 1984).

Tab. 1 — Limit values of high and toxic B concentrations in some crops (cit. Bergmann and Neubert, 1976)*

Crop	Growth stage at the time of sampling	Plant organ	Limit value (mg kg^{-1} in dry matter)		Reference
			High	Toxic	
Maize	Start of flowering	Leaf next to the ear	25—35	> 35	*Jones, 1967
Wheat	Tillering	Entire plant	31—100	> 100	*Finck, 1968
Potato	Start of flower	Basal and medium leaves	53—100	> 140	*Wrazidlo, 1973
Sugar beet	June/July	Lamina from rosette center	201—800	> 800	*Neubert et al., 1970
Bean	Start of flowering	Lamina of fully developed top leaf	> 150		*Chapman, 1967
Soybean	Before the start of pod forming	Fully developed top leaf	56—80	> 80	*Jones, 1967
Alfalfa	1st cut before flower	Entire aboveground part	53—99	> 99	*Gupta, 1972
Lettuce	Before harvest	Entire aboveground part	41—60	> 60	*Roorda van Eysinga et al. 1971
Sour cherry	July/August	Leaf	54	—	*Neubert et al., 1970
Grapevine	Flower/maturity	Leaf opposite to the grape bunch	40	—	*Levy, 1968
Maize	—	Leaves	—	100	El-Sheikh et al., 1971
Cucumber	—	Leaves	—	400	El-Sheikh et al., 1971
Squash	—	Leaves	—	1000	El-Sheikh et al., 1971
Wheat	—	Leaves	—	100—270	Paull et al. 1988
Snap bean	—	Leaves	—	100	François, 1989
Cow pea	—	Leaves	—	330	François, 1989
Picea sp.	—	Needles	—	960	Judel, 1977
Grasses	—	—	—	270—520	Judel, 1977

GENETIC BASIS OF B TOLERANCE

The only correct approach to increasing yields of crops grown on B-rich soils is the development of plant genotypes tolerant to B excess (Kraljević-Balalić et al., 2003). Recent physiological and genetic studies have contributed significantly to the understanding of the role of genetic variability in plant response to high B concentrations. Also, these studies facilitated the development of genotypes adapted to growing on soils high in B. Most of the crops found to possess large variability regarding the tolerance to B excess have the same tolerance mechanism — reduced B uptake.

Genetic variation for tolerance to B toxicity exists in a number of crops, including wheat and barley (Cartwright et al., 1987; Moody et al., 1988; Paull et al., 1988; Yau 2002, Torun et al., 2006), lentil (Yau and Erskine 2000), field pea and other forage crops (Paull et al., 1992). Huang and Graham (1990) stated that distinct and consistent differences among wheat genotypes in response to B toxicity, at both organ and cellular levels, could serve as a basis for breeding. The genotypes Evropa 90 (YUG), Peking 11 (CHI) and Kalayan Sona (IND), with lowest mean values of B concentration in leaves at heading stage, were appropriate sources for B tolerant germplasm. They may serve as B tolerant donors in hybridizations. Hexaploid wheat genotypes had, on average, higher B concentration in leaves at heading stage than tetraploid genotypes (Kraljević-Balalić et al., 2002).

Largest advances in the study of inheritance of tolerance to excess B have been registered in cereals. In wheat, this trait has been found to be coded for by at least four major genes, *Bo1, Bo2, Bo3* and *Bo4*. As these genes exhibit an additive action, there exists a broad array of tolerance levels. The occurrence of transgressive segregation in some crosses indicates that more than four genes may be involved in the control of this trait (Paull et al., 1993, Campbell et al., 1994). The wheat chromosomes that carry these genes are labeled as 4A, 7B, 7D and 7EB, which confirms the earlier hypothesis that the group 7 of homologous chromosomes play an important role in the control of tolerance to high B concentration. It is also probable that an allele on the chromosome 4D contributes to the increased sensitivity to B (Chantachume et al., 1994). As the wheat genotypes tolerant to B excess originate from various regions (Kraljević-Balalić et al., 2004) and as the transgressive segregants were found in the progenies of tolerant parents, it may be assumed that chromosomes other than those in groups 4 and 7 may carry genes that control the tolerance to excess B. A tolerant genotype, which outyielded sensitive ones by 50% when grown on B-rich soils, had been obtained by backcrossing the gene *Bo1* from a moderately tolerant genotype into a sensitive genotype which, on the other hand, was well adapted to other agroecological conditions (Campbell et al., 1994).

Using generation mean analysis, Kraljević-Balalić et al. (2004) found that the mode of inheritance of B concentration in wheat leaves in the F_1 and F_2 generations was intermediate, dominant or superdominant, depending on cross combination. B concentration was under the control of genes with additive, dominant and epistatic effects (a x a, a x d, d x d, respectively).

QTL analyses of barley showed that a major toxicity tolerance locus was located on the second arm of the chromosome 4H, while a moderate tolerance locus was on the chromosome 6H (Jefferies et al., 1999). To obtain reliable PCR markers for fine mapping of tolerance to excess B in all plant species, Schnurbusch et al. (2005) used the EST sequences from barley and wheat and genomic sequences identified in collinear regions of rice (*Oryza sativa* L., chromosomes 2, 3 and 6). These authors believe that the search through these EST data bases, together with the screening of a BAC library deriving from the lines tolerant to excess B, will enable a map-based isolation of such genes in barley and wheat, to determine ultimately the molecular mechanisms that ensure the tolerance to B excess in cereals.

Kaur et al. (2004) studied the genetic divergence of different canola genotypes (*Brassica rapa*) in hydroponic trials, field trials and by the method of molecular markers. The tolerant genotypes had significantly lower B contents than the sensitive ones, which confirmed that the tolerance mechanism is based on the capacity to avoid the uptake of high amounts of B. Analyses of genetic divergence by SRAP (Sequence Related Amplified Polymorphism) revealed that sufficient variability existed between the tolerant and sensitive genotypes for genome mapping of the B tolerance trait. The SRAP technique specifically targets coding sequences and results in the screening of co-dominant markers (Li and Quiros, 2001).

B tolerance exists also in some lentil genotypes (*Lens culinaris*), as reflected in their high yields when grown on soils rich in B. Application of these genotypes in breeding programs, particularly back crossing, produces significant results, especially when B tolerance is combined with tolerance to salts and diseases (Hobson et al., 2004). According to Bagheri et al. (1995), RAPD markers may be successfully used to characterize pea genotypes for genetic divergence regarding B tolerance.

Extensive studies have been conducted on arabidopsis (*Arabidopsis*) with the aim of identifying genes and proteins capable of providing tolerance to excess B. Five different types of genes have been found which, when expressed in yeast (a model eucariotic organism), exhibit tolerance to excess B (Fujiwara and Nozawa, 2005). An arabidopsis protein BOR1 has been found to transport B into the xylem. The amount of this protein regulates B transport from roots to aboveground parts. Under conditions of B shortage in the medium, it intensifies B transport to aboveground parts and under conditions of B excess it prevents excessive B accumulation in aboveground parts and toxic effects. It has been found that post-transcriptional mechanisms play a key role in the regulation of amounts of BOR1 (Takano et al., 2005).

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ФИЗИОЛОШКЕ И ГЕНЕТСКЕ ОСНОВЕ ТОЛЕРАНТНОСТИ БИЉАКА ПРЕМА СУВИШКУ БОРА

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Резиме

Како недостатак тако и сувишак бора (B) може значајно да смањи органску продукцију биљака, а у ексцесним случајевима може да доведе и до њиховог угинућа. Сувишак бора се пре свега јавља у аридним и семиаридним пределима, на заслањеним земљиштима или као последица активности човека. Ексцесне концентрације бора смањују раст биљака и изазивају физиолошке и морфолошке промене (хлорозу и некрозу) пре свега вршног и рубних или интеркосталних делова листа. Физиолошки механизми толерантности биљака према сувишку бора нису довољно познати. Преовладава мишљење да се они заснивају на рестрикцији усвајања и тиме накупљања B у корену и надземном делу биљака. Утврђено је да постоје значајне разлике у толерантности не само врста него и генотипова према сувишку B што омогућава да се путем оплемењивања створе генотипови гајених биљака подесни за гајење на земљиштима са повећаним садржајем приступачног B. С тим у вези интензивирају се истраживања у вези са наслеђивањем толерантности биљака према сувишку B. Утврђено је да код великог броја биљних врста (нпр. пшеница, слачица, грашак, сочиво, еукалиптус) постоје извори толерантности према сувишку B. Употребом различитих молекуларних техника које се заснивају на PCR-у (RAPD, SRAP) за анализе родитељских компонената и потомства настоји се да се што тачније мапирају гени носиоци толерантности према сувишку B. До сада је најбоље проучено наслеђивање овог својства код житарица. Утврђено је да код пшенице толерантност према сувишку B условљавају најмање четири гена, *Bo1, Bo2, Bo3* и *Bo4* који делују адитивно, тако да постоји читав спектар нивоа толерантности. Проучавања на арабидопсису довела су до бољег разумевања механизма регулације транспорта B из корена у надземне органе, што отвара могућност директнијих генетичких манипулација.

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GRAIN FILLING PARAMETERS IN HIGH-YIELDING NS WHEAT CULTIVARS

ABSTRACT: Grain yield of wheat is dependent on grain weight, which is the result of grain filling duration and rate. The study was undertaken to examine the relation between grain weight and rate and duration of grain filling in five high-yielding NS wheat cultivars. Stepwise multivariate analysis of nonlinear regression estimated grain filling parameters was used to examine cultivar differences in grain filling. On the basis of three-year average, the highest grain dry weight had cultivar Renesansa, and the lightest grains were measured for cultivar Evropa 90. Stepwise multivariate analysis indicated that all three nonlinear regression estimated parameters (grain weight, rate and duration of grain filling) were equally important in characterizing the grain filling curves of the cultivars studied, although sequence of their significance varied in different years, which is probably caused by different environmental conditions in three years of experiment.

KEY WORDS: grain filling, nonlinear regression, wheat

INTRODUCTION

Grain yield in wheat (*Triticum aestivum* L.) can be analyzed in terms of three yield components: number of spikes/m^2, number of grains/spike and grain weight. After anthesis, yield is largely dependent on final grain weight, which is the result of grain filling duration and rate (Whan et al., 1996).

The existence of genetic variation in wheat has been reported for both grain filling duration and rate (Darroch and Baker, 1995), and significant correlations have been found between grain weight and rate (Calderini and Reynolds, 2000), but also between grain weight and duration of grain filling (Evans et al., 1975). Gebeyehou et al. (1982) found significant correlations between both grain filling parameters (duration and rate) and yield.

Differences in relative importance of grain filling parameters for grain yield are probably caused by fact that environmental factors, especially temperature, also affect grain filling (Stone and Nicolas, 1994). A better understanding of the grain filling process may be helpful in breeding efforts to increase grain yield.

Linear regression (Van Sanford, 1985), quadratic (Nass and Reiser, 1975; Bruckner and Frohberg, 1987) and cubic equation (Gebeyehou et al., 1982) are statistical methods which have been used to describe grain filling in wheat. Univariate analysis of variance (ANOVA) can be used only to differentiate among grain growth curves and Darroch and Baker (1990) suggest stepwise multivariate analysis (Keuls and Garretsen, 1982) of nonlinear regression estimated parameters as more appropriate for analyzing growth curve parameters. Stepwise MANOVA can clarify the relative importance of the various parameters in a growth curve.

The objective of this study was to examine the relation between grain weight and rate and duration of grain filling in five high — yielding NS wheat cultivars.

MATERIAL AND METHODS

Five high-yielding NS wheat cultivars (Pobeda, Renesansa, Evropa 90, Sonata and Sofija) were chosen for this study in order to examine the possible differences in their grain filling pattern, and the relation between their grain filling parameters. It can point out the different ways for increasing yield in wheat.

The trial was conducted at the experimental field Rimski Šančevi, Institute of Field and Vegetable Crops, Novi Sad, in 2000, 2001 and 2002. The standard agrotechnic procedures were applied. Plot areas were 5 m^2, sown in four replications. Rimski Šančevi meteorological station data (temperature, precipitation) were used. Sampling started 14 days after anthesis and continued at 7-day intervals in first 3 weeks, and approximately 2-day intervals after, until maturity (13% moisture in grain). Random samples of 20 spikes per plot were harvested on each sampling date, selected in four replications. 10 grains from the middle of each of the 20 spikes were removed and oven dried at 80°C for 24 h. The grains were weighed before and after drying.

Dry matter accumulation over time and duration of grain filling were expressed as a function of accumulated growing degree days (gdd — °C) from anthesis. Growing degree days in particular sampling date is a sum of average daily temperatures from anthesis (Duguid and Brûlé-Babel, 1994). The grain weight and duration of dry matter accumulation data were fitted by nonlinear regression to a logistic curve: $y = W/(1+\exp(B-Cx))$ in order to calculate estimated grain filling parameters: final grain dry weight (W — mg), maximum rate (R — mg dry matter gdd^{-1}) and duration (T — gdd) of grain filling. Y is average grain weight (mg), x are gdd from anthesis, B is related to both duration and rate of grain filling and C is related to grain filling rate. The calculations are described in details in Darroch and Baker (1990). STATISTICA software package was used. Stepwise MANOVA described by

Keuls and Garretsen (1982) was used in order to determine which of the estimated parameters is the most important in characterizing the grain filling curves. The most significant parameter is one with the lowest Wilks' λ — value and the set can be extended to two or all three parameters. Only if the new parameter adds information not already contained in the set, its addition is considered to be important.

RESULTS AND DISCUSSION

The logistic curve provided a good fit to grain filling data in the study. In all 60 cases R^2 values exceeded 0.95, similar to results obtained by Duguid and Brûlé-Babel (1994).

Tab. 1 — Sum of temperatures (°C), average daily temperature (°C) and sum of precipitation (mm) in May and June in 2000, 2001. and 2002 (Rimski Šančevi meteorological station, Novi Sad)

Year	2000	2001	2002
Sum of temperatures	1214.5	1098.5	1245
Average daily temperature	20	18	20.4
Sum of precipitation	67	308	114

Univariate analysis of variance conducted on individual trials (years) showed significant differences among genotypes regarding all three (grain dry weight — W, rate — R and duration — T of grain filling) nonlinear regression estimated parameters in all three trials (Tab. 3). Stepwise multivariate analysis is used in order to determine the smallest set of estimated parameters that characterize the grain filling curves in each trial. Grain dry weight was the parameter with the smallest λ — value in 2000, therefore, of all three parameters, W was the most important in differentiating among grain filling curves. In 2001 the smallest λ — value is noted for parameter T, and for R in 2002 (Tab. 3).

Tab. 2 — Nonlinear regression estimated grain dry weight (W — mg), rate (R — mg dry matter $°C^{-1}$), duration (T — gdd) of grain filling and anthesis date (AD — number of days from 01. 01. to anthesis) in five high-yielding NS wheat cultivars, three-year trial

Cultivar	2000				2001				2002			
	W	R	T	AD	W	R	T	AD	W	R	T	AD
Pobeda	48.8	0.145	655	128	39.9	0.145	559	135	53.2	0.103	712	132
Renesansa	56.1	0.127	737	125	45.8	0.119	650	134	53.2	0.119	700	130
Evropa 90	50.5	0.130	684	128	39.8	0.116	618	135	47.4	0.099	711	130
Sonata	48.9	0.143	652	128	42.8	0.140	581	135	50.0	0.116	647	132
Sofija	50.1	0.125	711	127	44.0	0.106	706	136	50.3	0.113	680	132
Average	50.9	0.134	688	127	42.5	0.125	623	135	50.8	0.110	690	131

Variation in sequence of significance is probably the result of different environmental conditions in three years of experiment (Tab. 1). In all cases the

sets are extended to all three parameters (Tab. 4), which implies the significant impact of both grain filling duration and rate on grain weight.

Tab. 3 — Tests of significance of cultivar effects in MANOVA of final grain dry weight (W), maximum grain filling rate (R) and grain filling duration (T), measured in three trials (years)

Conditional set	df	2000		2001		2002	
		λ	F	λ	F	λ	F
W, R, T	12, 34	0.0121	12.46**	0.0027	24.28**	0.0006	43.31**
W, R	8, 28	0.0508	12.03**	0.0217	20.25**	0.0164	23.81**
W, T	8, 28	0.0655	10.18**	0.0221	20.05**	0.0099	31.75**
R, T	8, 28	0.0859	8.44**	0.0108	30.11**	0.0170	23.38**
W	4, 15	0.1495	21.34**	0.3250	7.79**	0.2156	13.64**
R	4, 15	0.3307	7.59**	0.0712	48.88**	0.1193	27.67**
T	4, 15	0.2222	13.13**	0.0515	69.00**	0.2602	10.66**

** — significant at the 0.01 level of probability
df — degrees of freedom
λ — Wilks λ criterion

Cultivar Renesansa had the highest grain dry weight in all trials, as a result of long grain filling with medium rate. On three-year average, the lowest grain filling rate and medium long grain filling of cultivar Evropa 90 resulted in the lightest grains of all five cultivars studied (Tab. 2). Thus, in our environments, the condition for heavy grains is not only long grain filling duration, but also the adequate balance between grain filling duration and rate. It is important to remark that cultivar Renesansa reached anthesis on three-year average two days earlier than other four cultivars (Tab. 2). Grain filling of Renesansa occurred in conditions different enough to provide gradual dry matter accumulation and to avoid terminal dry and temperature stress, in contrast to other cultivars studied.

Tab. 4. — Determination of the smallest set of variables required to completely characterize the grain filling curves in five high-yielding NS wheat cultivars

Year	Conditional set	λ	df	F	Final set
2000	W	0.1495	4, 15	21.34**	
	R/W	0.3398	4, 14	2.51^{ns}	
	T/W	0.4381	4, 14	1.79^{ns}	
	RT/W	0.0809	8, 26	8.21**	W, R, T
2001	T	0.0515	4, 15	69.00**	
	R/T	0.0108	4, 14	30.27**	
	W/TR	0.2500	4, 13	9.77**	T, R, W
2002	R	0.1193	4, 15	27.67**	
	W/R	0.1375	4, 14	5.94**	
	T/RW	0.0366	4, 13	86.04**	R, W, T

ns ** — nonsignificant, significant at the 0.01 level of probability
W — nonlinear regression estimated final grain dry weight
R — maximum rate of grain filling, T — grain filling duration
df — degrees of freedom
λ — Wilks' λ criterion

Variation among wheat genotypes regarding grain filling duration and rate indicates the possibility for breeding manipulation in purpose of increasing yields, however, other factors, such as anthesis date, number of grains per spike, number of spikes per m^2 and leaf area duration should also be considered.

CONCLUSION

The results of stepwise multivariate analysis of nonlinear regression estimated wheat grain filling parameters showed significant impact of both grain filling duration and rate on grain dry weight. The highest grain dry weight is noted for cultivar Renesansa, which was characterized by long grain filling with medium rate. Renesansa also reached anthesis two days earlier comparing to other cultivars studied.

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ПАРАМЕТРИ НАЛИВАЊА ЗРНА ВИСОКОПРИНОСНИХ НС СОРТИ ПШЕНИЦЕ

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Резиме

Принос пшенице зависи од масе зрна, која је резултат дужине и интензитета наливања зрна. Циљ рада је био испитивање веза између масе зрна и интензитета и дужине наливања зрна код пет високоприносних НС сорти пшенице. Stepwise мултиваријациона анализа нелинеарном регресијом процењених параметара наливања зрна је употребљена да се испитају разлике међу сортама у погледу наливања зрна. У трогодишњем просеку је највећу масу зрна имала сорта Ренесанса док су најлакша зрна измерена код сорте Европа 90. Stepwise мултиваријациона анализа је показала да су сва три нелинеарном регресијом процењена параметра (маса зрна, интензитет и дужина наливања зрна) једнако значајна за карактеризацију кривих наливања зрна проучаваних сорти, мада је редослед значајности варирао у различитим годинама. Овоме су вероватно узрок различити услови средине којима су проучаване сорте биле изложене током три године експеримента.

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EFFECT OF CYTOKININS ON THE ACTIVITY OF SUPEROXIDE DISMUTASE IN NITROGEN DEFICIENT WHEAT

ABSTRACT: Reactive oxygen species (ROS), such as $O_2 \cdot^-$, are formed by electron transfer to a molecule with stable electron configuration, in electron transport chains in the cell. ROS are very reactive molecules which are formed at higher rates under stress, such as drought, high insolation, heath, inadequate mineral nutrition, and such conditions lead to impairment of various physiological and biochemical processes in the cell. To reduce production of ROS, and their detrimental effect, plants developed various enzymatic and non-enzymatic protective mechanisms. Superoxide dismutase (SOD) is one of the most important antioxydative enzymes, which removes superoxid anion radical ($O_2 \cdot^-$), whose rate of production is the highest under unfavorable environmental conditions. Plant tissues that exhibit delayed senescence often have higher cytokinin content, which is accompanied by reduced amount of ROS. The focus of this paper is to examine whether foliar application of cytokinins to young wheat plants insufficiently supplied with nitrogen affects the activity of SOD and amount of $O_2 \cdot^-$. Application of trans-zeatine (CK) reduced the activity of SOD, but this reduction was not accompanied by an increase in the amount of $O_2 \cdot^-$. Application of benzyl adenine (BA) also reduced the activity of SOD, with concomitant increase in the amount of $O_2 \cdot^-$ in wheat leaves.

KEY WORDS: wheat, nitrogen supply, cytokinin, trans-zeatine, benzyl adenine, superoksid-dismutase, superoxid anion radical

INTRODUCTION

Reactive oxygen species (ROS) are extremely reactive molecules because they have unpaired electron in their structure. ROS formation takes place in all parts of the cell, in which electron transport takes place (Elstner, 1991). Superoxide anion radical ($O_2 \cdot^-$), one of the ROS, is formed by electron transfer to molecular oxygen (O_2) (Mehler, 1951): $\bullet (e) + O_2 \rightarrow O_2 \cdot^-$. This reaction takes place during the electron transport processes in a number of cell com-

partments. The highest amount of $O_2 \cdot^-$ in plant cells are formed in chloroplasts, mitochondria and peroxisomes (Fridovich, 1986).

Superoxide anion radical ($O_2 \cdot^-$) is very reactive molecule which acts as progenitor of destructive chain reactions that finally result in the damaging of the molecules close to the site of $O_2 \cdot^-$ production (Berlett and Stadtman, 1997). Therefore, keeping the low cellular level of $O_2 \cdot^-$ is of crucial importance for cell metabolism. To do that, plant cells developed a number of efficient biochemical mechanisms during the evolution. The most efficient mechanism for removal of $O_2 \cdot^-$ from the cell is "ascorbate-glutathione" cycle, which functions in chloroplasts, mitochondria and peroxisomes, where the highest amount of $O_2 \cdot^-$ is formed (Asada, 1999).

The first reaction in the "ascorbate-glutathione" cycle is transformation of two molecules of superoxide anion radical ($O_2 \cdot^-$), with two protons (H^+), into one molecule of hydrogen peroxide (H_2O_2) and one molecule of oxygen (O_2). The enzyme that catalyses this reaction is superoxide dismutase (SOD):

$$O_2 \cdot^- + O_2 \cdot^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

The importance of SOD in the removal of $O_2 \cdot^-$ is thoroughly proven (Perl et al., 1993; Yu et al., 1999). Plants with increased synthesis of SOD in their chloroplasts had higher tolerance to stress-inducing factors such as drought, low or high temperatures, imbalanced mineral nutrition, etc. Under stress, in plants with higher SOD activity the growth was less reduced than in plants in which SOD activity did not increase upon the induction of stress (Štajner et al., 2004). Therefore, the activity of SOD may be used as a parameter to evaluate the level of plant tolerance to particular stress-inducing factor.

Cytokinins can also participate in removal of ROS from the cell. After Leshem et al. (1979) the molecular structure of cytokinins allows them to react directly with superoxide anion, but also with the other ROS, thus removing them from the cell metabolism.

As the possible direct antioxidant activity effect of cytokinins can also be considered their effect on the activity of antioxidant enzymes, including SOD. While studying the effects of cytokinins on senescence, several authors (Grossman and Leshem, 1978; Liu et al., 1996) found that cytokinins, such as zeatin and benzyl adenine, can increase the activity of some antioxidative enzymes (SOD, catalase), and therefore delay senescence of the plant tissue. Moreover, Leshem et al. (1981) showed that cytokinins inhibit the activity of xanthine oxidaze, an enzyme that is one of the generators of ROS in the cell. The senescence was delayed also by reduction of lipoxygenase activity induced by cytokinins, which contributed to preserve the integrity of cell membranes.

Although it is known that cytokinins have the potential to reduce oxidative stress in plants, this was not tested under nitrogen starvation in wheat. Therefore, the aim of this study was to analyze the effect of foliar application of cytokinins (zeatine and benzyl adenine) on the activity of SOD and amount of $O_2 \cdot^-$ in the leaves of young nitrogen-deficient wheat plants.

MATERIALS AND METHODS

The effect of cytokinins on the activity of SOD and amount of $O_2 \cdot^-$ was analyzed in bread wheat (*Triticum vulgare*), cultivar Renesansa, differently supplied with nitrogen. Young wheat plants were grown in water cultures and foliary treated with solutions of trans-zeatine (CK) and benzyl adenine (BA), under semi-controlled conditions.

Plant growth

Germination was done in an incubator, in the dark, at 26°C. Seedlings were divided into three groups at planting. One was supplied with the complete ½ strength Hoagland nutrient solution (H o a g l a n d and A r n o n, 1950) (control, N1, nitrogen supplied as [2.5 mM KNO_3 and 2.5 mM $Ca(NO_3)_2$]), another onto the same solution but with the N concentration reduced to ½ (N1/2, nitrogen supplied as [1.25 mM KNO_3 and 1.25 mM $Ca(NO_3)_2$]), and for the third group N concentration was reduced to ¼ of the full dose (N1/4, [0.625 mM KNO_3 and 0.625 mM $Ca(NO_3)_2$]). Nutrient solutions were aerated each day and replaced every 3—4 days. Fifteen days after planting, each group was divided into 3 sub-groups of 6 pots and 8 plants per pot each, and treated with solutions of trans-zeatine (CK) and benzyl adenine (BA).

Treatment with cytokinins

Plants were treated with water solution of trans-zeatine (CK) of the following concentrations: 0 mg/dm^3 (deionized water-control, CK 0), 2.5 mg/dm^3 (CK 2.5), 5,0 mg/dm^3 (CK 5) and 10 mg/dm^3 (CK 10). Benzyl adenine (BA) was applied at following concentrations: 0 mg/dm^3 (deionized water-control, CK 0), 10 mg/dm^3 (BA 10), 40 mg/dm^3 (BA 40). Foliar treatments with both CK and BA were done twice during the experiment by spraying 0.1 dm^3 of each solution of 144 plants. The first treatment was done 7 days after planting and the second 14 days after planting. Plants were analyzed 7 days after the second treatment.

Extraction of $O_2 \cdot^-$ and SOD

$O_2 \cdot^-$ and SOD were extracted from 1 g of fresh leaf tissue, in phosphate buffer pH 7.0, as described by Q u y H a i et al. (1975).

Determination of the amount of O_2^- and the activity of SOD

Superoxide radical ($O_2 \cdot^-$). The amount of $O_2 \cdot^-$ was determined by the method of M i s r a and F r i d o v i c h (1972), based on the reaction of inhibition of the auto oxidation of adrenaline.

Superoxide dismutase (SOD). The total activity of SOD was determined on the basis of the inhibition of transformation of adrenaline into adrenochrome in the presence of the air (M i s r a and F r i d o v i c h, 1972).

Statistical analysis

The results were statistically processed by the analysis of variance, and calculation of the least statistically significant differences (LSD) between the control and cytokinin treatment, for each level of nitrogen nutrition and for probability of 5%, using the computer program STATISTICA 7.

In the figures, in the Results section, numerical values above the bars represent relative (in %) increase or decrease of the measured value of each treatment with respect to the control (control = 0). The control plants are those grown on complete ½ strength Hoagland solution and untreated with cytokinins (N1, CK 0, BA 0).

Following the ranking of mean values by the Duncan test, the significance of differences between the means is marked by letters inside the figure bars. Two means differ significantly (for $p < 0.05$) if they do not share any letters.

RESULTS

The effect of cytokinins on the activity of SOD

The activity of SOD ranged from 33.57 U/g FW (in treatments N1/2 and N 1/4, CK 10) to 205.52 U/g FW (in the control). Such a high span between measured values suggests that both nitrogen nutrition and treatment with cytokinins affected the activity of SOD (Fig. 1).

An increase in N concentration in the nutrient solution increased the activity of SOD, whereas increase in CK concentration significantly reduced activity of SOD in young wheat leaves. Analysis of variance permits to conclude

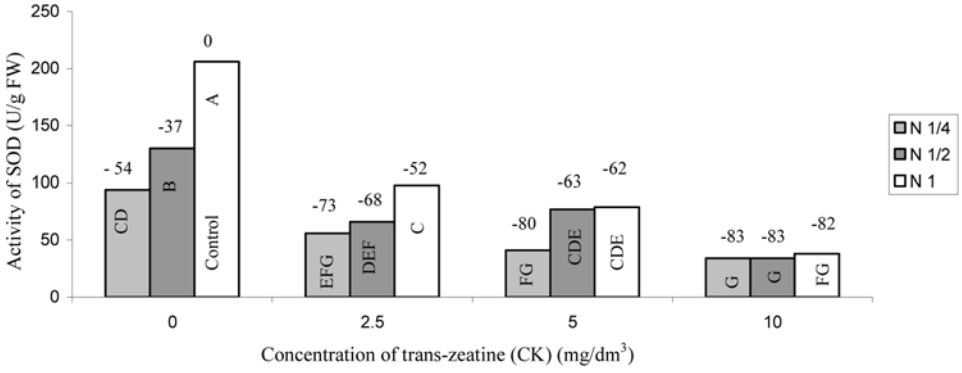

Fig. 1 — Activity of superoxide dismutase (SOD) in leaves of young wheat plants grown in the presence of different N concentrations and treated with different concentrations of CK

that there is an interaction between applied N and CK concentrations on the activity of SOD. The effect of N concentration on the activity of SOD significantly varied with the increase in the concentration of CK.

In the experiment with BA, the highest activity of SOD was found in the control (N1 and BA0 — 186.05 U/g FW). The lowest activity was found in plants supplied with the lowest N and the highest BA concentration (N1/4 and BA40 — 26.00 U/g FW) (Fig. 2).

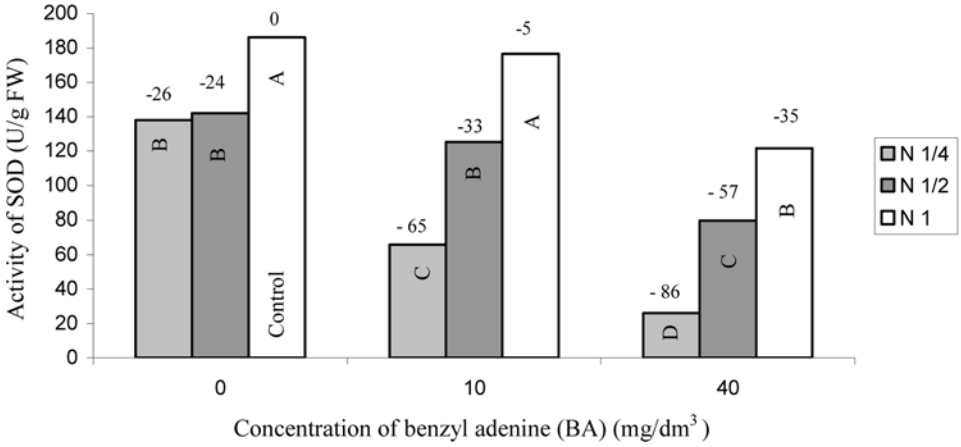

Fig. 2 — Activity of superoxide dismutase (SOD) in leaves of young wheat plants grown in the presence of different N concentrations and treated with different concentrations of BA

With the reduction of N concentration in the nutrient solution the activity of SOD declined as well. On the contrary, an increase in the BA concentration reduced the activity of SOD. However, analysis of variance showed that there was no statistically significant interaction between N and BA concentration on the activity of SOD.

The effect of cytokinins on the amount of superoxide radical ($O_2 \cdot^-$)

In the experiment with CK, the highest amount of $O_2 \cdot^-$ (4.35 mmol/g FW) was found in plants supplied with ¼ of full dose of N and treated with 2.5 mg CK/dm^3 solution, whereas the lowest amount of $O_2 \cdot^-$ (2.64 mmol/g FW) was found in plants supplied with full dose of N and treated also with 2.5 mg CK/dm^3 solution (Fig. 3).

The concentration of N in the nutrient solution exhibited statistically significant effect on the reduction of the amount of $O_2 \cdot^-$ in wheat leaves. This reduction was the most outstanding in plants grown in the presence of full N dose (N1) (Fig. 3). The effect of treatment with CK on the amount of $O_2 \cdot^-$ in wheat leaves was not statistically significant.

Treatment with BA significantly increased the production of $O_2 \cdot^-$ in leaves. The highest amount of $O_2 \cdot^-$ (4.68 mmol/g FW) was found in leaves of wheat

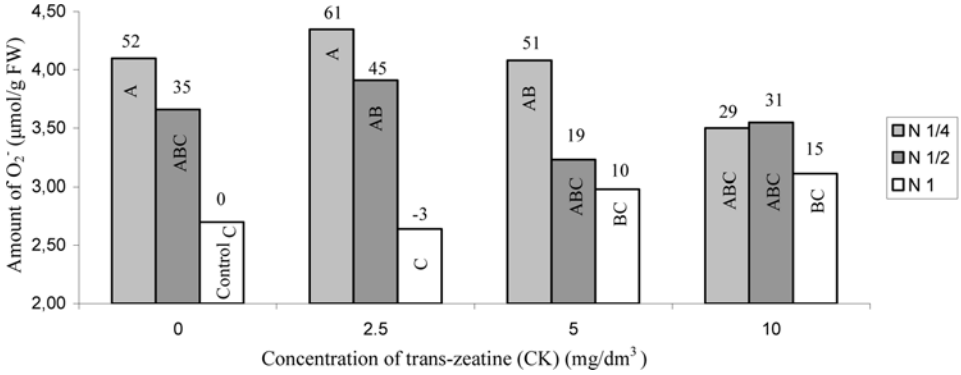

Fig. 3 — Amount of superoxide radical ($O_2 \cdot^-$) in leaves of young wheat plants grown in the presence of different N concentrations and treated with different concentrations of CK

grown on nutrient solution containing ¼ of full dose of N and treated with solution containing 40 mg BA/dm^3. At the same time, in control plants only 2.14 mmol $O_2 \cdot^-$/g FW were detected (Fig. 4).

Both alteration of N and BA concentration significantly affected the production of $O_2 \cdot^-$ in wheat leaves. Analysis of variance showed that an increase in N concentration, contrary to BA, reduced the amount of $O_2 \cdot^-$. Moreover, significant interaction between effects of N and BA was present.

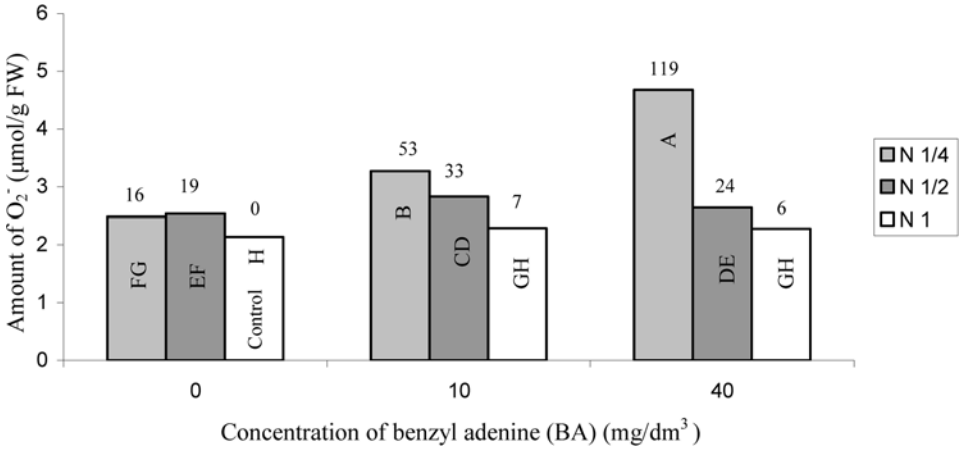

Fig. 4 — Amount of superoxide radical ($O_2 \cdot^-$) in leaves of young wheat plants grown in the presence of different N concentrations and treated with different concentrations of BA

DISCUSSION

There is a negative correlation between measured activities of SOD and the determined amounts of $O_2 \cdot^-$ in plants treated with different concentrations of BA (Fig. 2, Fig. 4), which is in accordance with the results of A s c h l e r

et al. (2002). However, in the plants treated with CK, there is an absence of the correlation between the activity of SOD and the amount of $O_2 \cdot^-$ (Fig. 1 and Fig. 3). In other words, in the plants treated with CK significant reduction in SOD activity was not accompanied by significant increase in the amount of $O_2 \cdot^-$. This may be explained by the role of trans-zeatine as the signal molecule in the plants. For example, Takei et al. (2002) found that the synthesis of cytokinins in root tips and their concentration in the plant tissue increased with an increase in the concentration of nitrate N in the nutrient substrate. High concentrations of nitrate in the soil substrate are typical for conditions of sufficient content of organic matter in the soil, optimal temperature and moisture in the soil and in such conditions the synthesis of cytokinins is stimulated. Concomitantly, the transport of cytokinins through xylem is also accelerated and this represents the signal to a plant that nutritive and moisture conditions are favorable for growth.

Because the biosynthesis of cytokinins is stimulated when environmental conditions are favorable for plant growth and development, it is highly probable that binding of cytokinins for specific receptors in the cell membranes induces the production of a signal that triggers the synthesis of enzymes that allow plants to utilize as much as possible favorable environmental conditions. This assumption is supported by experimental results of Chen and Leisner (1985), Chen (1989) and Andersen et al. (1996), who, after application of cytokinins, found an increase in activity of Rubisc/o, fructose-1,6--diphosphatase, glyceraldehyde-3-phosphate dehydrogenase, NADP-dependant malate dehydrogenase, hydroxypyruvate reductase and nitrate reductase in plant tissues.

In addition, under favorable environmental conditions, accompanied by higher cytokinin content, the need for antioxidant enzymes is reduced. Therefore, it is probable that under the experimental conditions described in this paper treatment with CK induced transient increase in cytokinin concentration in leaf tissues, which resulted in the reduced synthesis of antioxidant enzymes, including SOD. Similar result was described by Crowell and Amasino (1991). In cell culture of soybean cells, isolated from seedlings, and grown on a medium without addition of cytokinins the synthesis of FeSOD increased, while addition of cytokinins reduced the level of mRNA corresponding to gene encoding FeSOD. On the contrary, He et al. (2005) found that in hybrid corn exhibiting delayed senescence an increase in cytokinin content is accompanied by an increase in SOD activity. Moreover, Liu and Huang (2002) found an increase in SOD activity following addition of zeatin-riboside to the rhizosphere of the grass *Agrostis palustris*, which was exposed to combined stress provoked by high temperatures of the soil and the air. Durmus and Kadioglu (2005) treated maize leaves with 2.5 and 25 mg BA/dm^3 and recorded an increase in the activity of SOD if plants were treated with paraquat 8 h after the BA treatment, but if paraquat was applied 12 or 24 h after BA there was no increase in the activity of SOD.

So variable effects of different cytokinins on the activity of SOD can be explained by the fact that the activity of SOD depends, among the other factors, on the plant and tissue age, complexity of environmental factors, partitio-

ning between enzymatic and non-enzymatic antioxidants in the plant, and on the nature and intensity of the eventual stress factor(s).

CONCLUSIONS

The effects of foliar treatment of nitrogen-deficient young wheat plants with CK and BA on activity of SOD and amount of $O_2 \cdot^-$ were studied under semi-controlled conditions. On the basis of the experimental results, the following conclusions can be drawn out:

Foliar application of CK and BA significantly reduced the activity of SOD in the leaves of nitrogen-deficient young wheat plants.

Foliar application of BA increased the amount of $O_2 \cdot^-$ in leaves of nitrogen-deficient young wheat plants while treatment with CK did not effect the amount of $O_2 \cdot^-$.

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УТИЦАЈ ЦИТОКИНИНА НА АКТИВНОСТ СУПЕРОКСИД-ДИСМУТАЗЕ ПШЕНИЦЕ У УСЛОВИМА НЕДОСТАТКА АЗОТА

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Резиме

Слободни кисеонични радикали (ROS) настају принудним преносом електрона са неког од многобројних електрон-транспортних ланаца у ћелији, на молекуле са стабилном конфигурацијом електронског омотача. Настали радикали су веома реактивни молекули који се у већој количини стварају у стресним условима (висока осветљеност, суша, недовољна исхрана), који доводе до поремећаја физиолошко-биохемијских процеса у ћелији. Да би се смањило настајање радикала и умањило њихово штетно дејство, у биљкама су се током еволуције развили ензимски и неензимски системи заштите. Супероксид дисмутаза (SOD) је један од најважнијих антиоксидантних ензима који уклања настали супероксид анјон радикал ($O_2 \cdot^-$), чије је настајање најинтензивније у неповољним условима спољашње средине. Уочено је да је једна од карактеристика биљних ткива, чије је старење одложено, да је повећан садржај цитокинина у биљном ткиву праћен смањењем количине кисеоничних радикала. На основу ове чињенице одређен је и циљ овог рада, да се, у условима недостатка азота, утврди утицај третирања младих биљака пшенице растворима једињења са цитокининском активношћу на активност SOD и количину $O_2 \cdot^-$. Третирање пшенице растворима транс-зеатина (CK) смањило је активност SOD, али то смањење није праћено повећањем количине $O_2 \cdot^-$. Примена раствора бензил аденина (BA) је такође довела до смањења активности SOD, али је ово смањење активности праћено повећањем количине $O_2 \cdot^-$.

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ORAL CANDIDIASIS-ADHESION OF NON-ALBICANS *CANDIDA* SPECIES*

ABSTRACT: Oral candidiasis is an opportunistic infection caused primarily by *Candida albicans*. However, in recent years, species of non-albicans *Candida* have been implicated more frequently in mucosal infection. *Candida* species usually reside as commensal organisms and are part of normal oral microflora. Determining exactly how transformation from commensal to pathogen takes place and how it can be prevented is continuous challenge for clinical doctors. Candidal adherence to mucosal surfaces is considered as a critical initial step in the pathogenesis of oral candidiasis. Acrylic dentures, acting as reservoirs, play an important role in increasing the risk from *Candida* colonisation. Thus, this review discusses what is currently known about the adhesion of non-albicans *Candida* species of oral origin to buccal epithelial cells and denture acrylics.

KEY WORDS: Adhesion, antifungals, buccal epithelial cells, *Candida* carriage, denture acrylic surface, non-albicans *Candida* spp.

INTRODUCTION

Oral candidiasis is a common opportunistic infection both in immunocompromised and otherwise healthy individuals. *Candida albicans* is the most frequently isolated pathogenic member of the genus *Candida* (Meyer et al., 1998). However, in recent years, as a consequence of the extensive use of azole drugs, such as fluconazole, species of non-albicans *Candida*, such as *C. glabrata*, *C. krusei* and *C. parapsilosis*, have been implicated more frequently in mucosal and sistemic infections (Krcmery and Barnes, 2002). *Candida glabrata* has emerged as a notable pathogenic agent in the oral mucosa, frequently being coisolated with *C. albicans* or the only detectable species. This is particularly important because *C. glabrata* isolated from oral lesions is much more resistant to antifungal treatment than *C. albicans* (Redding, 2001). An additional recent development is the recognition of new species associated with human pathology, such as *C. dubliniensis* (Gutierrez et al.,

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2002). *Candida dubliniensis* is a recently described species first isolated from oral lesions in HIV-infected individuals (Schorling, 2000).

CANDIDA CARRIAGE

Candida spp. form a part of the normal oral flora and is present in at least 50% of the population. Reports of oral carriage of *C. albicans* vary greatly in the literature, although most investigators agree that yeast are commonly found in the mouths of healthy persons and that a significant percentage of the species found are *C. albicans*. A compilation of data from a number of reports showed that oral carriage rate in healthy individuals was from 35 to 80% (Ben-Aryeh et al., 1995). It is difficult to give a precise oral carriage rate for *C. albicans*, since this depends on the age and health of the studied population and used sampling methods (White et al., 2004). *Candida* carriage is more frequent in women, persons of blood group 0, denture wearers, smokers, immunocompromised persons and hospitalized patients. Also, the high carbohydrate diet, xerostomia and use of broad-spectrum antibiotics increase the possibility of *Candida* carriage (Scully, 2004). Mucosa of the tongue dorsum may represent a site of residual colonization and a reservoir of organisms. In healthy individuals, *C. albicans* is most commonly isolated from the mid-line of the middle and posterior thirds of the tongue (Epstein et al., 2001).

HOST DEFENCES

In the mouth, epithelial physical barrier, indiginous of saliva, salivary IgA, lyzozyme, histidine-rich polypeptides, lactoferrin, lactoperoxidase seem to play an important role in keeping *Candida* under control (Jorge et al., 1993). *Candida* elicits both humoral and cell mediated immune response (CMI) in a mammalian host. CMI is a predominant defence system against *Candida* during infection, as well as under asymptomatic carriage, and can be detected by *in vitro* and *in vivo* assays. Cytokines, such as interleukin (IL) IL-2, IL-12, THF-alpha and IFN-gamma seem to be of importance in the defence system (for more extensive discussion see review by Dongari-Bogtzoglou and Fidel, 2005). Anti-Candida antibodies of all imunoglobulin types can be detected in experimental and natural infections and in healthy humans carrying *Candida* (Segal, 2005). Anti-Candida sIgA can be detected in saliva, and its concentration is increased in whole or parotid saliva from HIV-positive individuals, but reduced in AIDS patients, suggesting that a compensatory response is overcome with progressive immunodeficiency (Challacombe and Sweet, 1997).

PREDISPOSING FACTORS

The transition from harmless commensal to unrelenting pathogen is dependent not only on virulance factor of the organisms but also equally, or even more, on host factors. The presence of such predisposing factors, both local

and systemic (Table 1), is important since it has been extremely rare to find a case of oral candidiasis in which one or more of these factors cannot be identified (reviewed in Scully et al., 1994).

Table 1. Predisposing factors for oral candidiasis

• Xerostomia (irradiation, Sjögren's syndrome, xerogenic drugs, cytotoxic drugs)
• Smoking
• Changes in oral microbial flora (broad-spectrum antibiotics, corticosteroids, dentures)
• Physiological (infancy, pregnancy, old age)
• Endocrine disorders (Diabetes mellitus, Addison's disease, hypothyroidism)
• Malnutrition (high-carbohydrate diet, iron, folate, vitamin B12 deficiencies)
• Malignancies (leukemia, agranulocytosis)
• Immune defects (HIV infection, AIDS, transplantation)

THE ADHESION OF NON-ALBICANS *CANDIDA* SPECIES

Candida species have developed an effective battery of virulence factors and specific strategies to assist in their ability to colonize host tissues, cause disease and overcome host defences. The virulence factors expressed by *Candida* species causing infections may well vary depending on the type of infection, the site and stage of infection, and the nature of the host response.

The adhesion of *Candida* to host mucosal surfaces is a vital prerequisite for successful colonisation and infection. Attachment enables the organisms to avoid dislodgement by cleansing action of mucosal secretions, and it facilitates infection. It has been shown that the yeast cell wall components, capable of interacting with a variety of ligands on the host cell surface, including proteins are carbohydrates, are important constituents of the adhesion process (reviewed by Chaffin et al., 1998, Cannon and Chaffin, 1999). Oral cavity presents a number of surfaces for candidal adhesion. The adhesion to buccal epithelial cells (BECs) and denture acrylic surfaces will be discussed in this review.

THE ADHESION TO BUCCAL EPITHELIAL CELLS

There are numerous reports concerning the adherence ability of *C. albicans*, and the readers are referred to review articles which summarize the most relevant data available (Cannon and Chaffin, 1999). However, only a few studies evaluate the adherence ability of other *Candida* species. Samaranayake et al. (1995) demonstrated a positive correlation between the surface hydrophobicity of *C. kruzei* and *C. albicans* and their adherence to BECs. Ellepola et al. (1999) did not find significant differences in the adherence ability between *C. albicans, C. glabrata, C. parapsilosis* and *C. tropicalis*, and Lyman et al. (1999) demonstrated a hierarchy in adherence in which *C. glabrata* and *C. krusei* adhered in greater number to rabbit esophageal mucosa than fluconazole-susceptible species. Differently, in a study made by Bia-

s o l i et al. (2002), *C. albicans* was significantly more adherent to BECs than *C. glabrata, C. krusei* and *C. lusitaneae*. In the recent study, *C. albicans* adhered to BECs in a greater number, followed by *C. tropicalis*, *C. glabrata* and *C. parapsilosis* with a significant difference in adhesion between the species, except for *C. glabrata* and *C. parapsilosis*. *Candida glabrata*, *C tropicalis* and *C. parapsilosis* strains obtained from the oral cavity of denture wearers with signs of denture stomatitis were able to adhere to BECs in a higher intensity than the isolates obtained from patients with normal palatal mucosa (L y o n and d e R e s e n d a, 2006). T o b g i (1989) used five isolates of *C. parapsilosis* to demonstrate the hierarchy of adherence to BECs among six species of *Candida*. He found *C. albicans* to be the most adherent, followed by *C. tropicalis, C. parapsilosis, C. glabrata, C. guilliermondii* and *C. krusei*. However, P a n a g o d a et al. (2001) investigated *in vitro* adherence of 24 isolates of *C. parapsilosis* and 12 isolates of *C. albicans*, and found no significant intraspecies difference in the adhesion of both species to BEC, although the former demonstrated a tendency for increased adherence. Analysis of the data reveals that such apparent differences do not reach significant levels due to large spread of the adhesion values. The apparent increase in the number of adherent *C. parapsilosis* noted may also be a reflection not only of yeast cell to epithelial cell adherence, but also of high co-adherence between organisms. The latter type of interaction results in the formation of yeast aggregates on the epithelial cell surface, which was more often observed with *C. parapsilosis* to BECs and that could be attributed to variation in the strains and culture condition. In the same study, P a n a g o d e et al. (2001) observed a significant intraspecies variation in adherence to BECs of *C. parapsilosis* isolates. An analogous phenomenon was documented by S a m a r a n a y a k e et al. (1995), where a significant intraspecies variation in adherence to BECs by 20 isolates of *C. krusei* was demonstrated. There is only a single study indicating the relationship between the source of the isolate and adherence of *C. parapsilosis* to BECs. On investigation of the adherence results of superficial and systemic isolates of *C. parapsilosis*, the former demonstrated a tendency for higher adherence to BECs than the systemic isolates. Although no significant difference was not noticed between these two groups, the superficial isolates demonstrated 51,5% more avidity for BECs than the systemic counterparts (P a n a g o d a et al., 2001).

Despite the availability of a spectrum of antifungals for the treatment of oral candidiasis, therapy failure is observed frequently. The diluent effect of saliva and cleansing effect of the oral musculature may reduce the level of antifungals below their effective therapeutic concentrations (M a r t i n, 1990). Thus, during topical treatment, the yeast undergoes exposure to a relatively brief antifungal agent and the drug concentration is likely to vary in different niches of the mouth. Moreover, the formation of *Candida* biofilms on oral surfaces may also contribute to a failure of drug therapy (H a w s e r and D o u g l a s, 1995). Nystatin belongs to the polyene group of antimycotic agents and is widely used as a topical agent in the management of oral candidiasis. There is only a single report on the adhesion of 30 oral isolates of *Candida* belonging to six different species (comprising *C. albicans, C. tropicalis, G. glabrata,*

C. guilliermondii, C. krusei and *C. parapsilosis*), to human BECs, following their brief exposure (1h) to a minimum inhibitory concentration of nystatin. Nystatin induced suppression of adhesion was the least for *C. albicans* (53,85%) compared with the other five species (64,09—67,74%). However, such significant intraspecies difference could not be elicited amongst the other five *Candida* species (Ellepola et al., 1999). In clinical terms these results demonstrated that exposure to nystatin significantly reduces candidal adherence to BECs irrespective of the *Candida* species concerned. The subtherapeutic levels of antimycotics likely to persist in the oral cavity during dosing intervals may be beneficial in reducing candidal colonisation, though possibly ineffective in their total elimination. Ellepola and Samaranayake (1999) measured the post-antifungal effect (PAFE) of 30 oral isolates of six different *Candida* species, and found that nystatin-elicited PAFE was lowest for *C. albicans* and greatest for *C. parapsilosis*, while *C. krusei, C. tropicalis, C. glabrata* and *C. guilliarmondii* elicited intermediate values. These findings clarify another possibility for the persistent, chronic recurrence of oral *C. albicans* infection despite apparently adequate antifungal drug regiments. It seems that even a limited exposure to the minimum inhibitory concentration of nystatin would confirm the growth suppression of non-albicans species.

There are only a few studies evaluating the action of fluconasole in the adherence ability of other *Candida* yeast. Darwazeh et al. (1991), in a study involving four dentate healthy subjects, found a significant reduction in *C. albicans* adhesion to BECs after a week of fluconazole intake. Braga et al. (1996) found that fluconazole in subinhibitory concentration was inactive to interfere in the adhesion ability of *C. glabrata*. Furthermore, a reduction in the adherence ability of *C. glabrata, C. tropicalis* and *C. parapsilosis* to BECs was found after exposure to fluconazole, both among the isolates obtained from the denture wearers with sign of oral candidiasis, and the isolates obtained from the denture wearers with normal palatal mucosa, even considering that *C. glabrata* frequently shows high minimum inhibitory concentrations to fluconazole (Lyon and de Resende, 2006). These results suggest that the adhesion, even of non-albicans species, could be factor that, along with predisposing conditions related to the host, determines whether an individual will develop disease or remain as a healthy carrier and confirm that fluconazole has an impact on the adherence ability of *Candida* spp. Dorocka-Bobkowska et al. (2003), using *C. albicans* and *C. glabrata* isolates obtained from the oral cavity with denture stomatitis, found that the incubation of human epithelial cell and human squamous cell carcinoma HSC-3 cells with both *Candida* spp., in the presence of amphotericin B, nystatin or natamycin, reduced the candidal adherence to these cells. When compared to amphotericin B, nystatin and natamycin suppressed the adherence less effectively, and these differences were statistically significant. Also, candidal adherence was significantly reduced when the tested polyenes were present during the "adherence phase". These findings suggest that subtherapeutic levels of polyenes, that are likely to persist in the oral cavity following topical treatment may modulate candidal colonisation when present during the "adherence phase".

THE ADHESION TO DENTURE ACRYLIC SURFACES

The adhesion of *C. albicans* to denture acrylic surfaces, and the ability to promote colonisation and infection in the oral cavity have been investigated in a number of studies (reviewed in Chaffin et al., 1998). However, there have been a few studies on the adhesion of non-albicans *Candida* species, and all have used *C. albicans* as the test organisms. In one study, the adhesion of *C. albicans* and *C. tropicalis* to 21 denture base material was investigated, and the adherence of *C. albicans* in general was far inferior to that of *C. tropicalis* (Minagi et al., 1985). Furthermore, the isolates of *C. krusei*, and emerging pathogen, showed variable but greater hydrophobicity than *C. albicans* isolates, and there was no correlation between hydrophobicity and adherence to denture acrylic (Samaranayake et al., 1995).

There have been few studies on the adhesion of *C. glabrata* isolates to acrylic surfaces with contradictory findings (Miyake et al., 1986, Hazen et al., 1986, Minagi et al., 1986, Klotz et al., 1985). The reason for these contradictory findings could be the fact that the above studies used limited number of *C. glabrata* (up to 6 species). Therefore, Luo and Samaranayake (2002) studied a battery of 34 oral isolates of *C. glabrata* and 15 oral isolates of *C. albicans*, with respect to their relative cell surface hydrophobicity (CSH) and adhesion to denture acrylic surfaces. Their results indicated a remarkable intraspecies differences in both CSH and the adhesive ability of *C. glabrata* strains. Compared with *C. albicans, C. glabrata* demonstrated a four-fold greater CSH value and a two-fold greater tendency to adhere to denture acrylic surfaces. They have also noted a highly significant positive correlation between the relative CSH and adhesion of *C. glabrata*. This implies that the higher hydrophobicity of isolates, the greater tendency to adhere to acrylic surfaces. A significant positive correlation was also noted between the relative CSH and adhesion of 24 isolates of *C. parapsilosis* to acrylic surfaces confirming the interrelationship between these pathogenic attributes (Panagota et al., 2001). These data substantiated the close relationship between the relative CSH and adhesion of *Candida* spp. In this relationship there may exist some yet unrealized changes in the surface free energy which entail the process of attachment (Minagi et al., 1986, Gerson and Akit, 1980). It is also likely that the phenomenon of co-adhesion between closely apposed blastoconidia, particularly of *C. glabrata*, may contribute, since hydrophobic cells exhibit a higher tendency to co-adhere than their hydrophobic counterparts.

The adhesion of *C. albicans, C. glabrata, C. krusei* and *C. dubliniensis* to heat-cured acrylics (Vetex™ Rapid Simplified and ProBase™ Hot) and cold--cured acrylics (Paladur® A and Paladur® B) was investigated and the most important finding was the difference in yeast adherence between Vertex™ and the other acrylics. Only *C. glabrata* species attached to Vertex. All four species tested attached to all the remainder of the tested acrylics, except ProBase™ Hot, which could not sustain the adherence of *C. krusei*. Also, there were significant differences in the adhesion of *C. albicans, C. glabrata* and *C. krusei* between heat-cured acrylics and cold-cured acrylics (He et al., 2006). These data indicate that candidal adhesion to denture base acrylics differ de-

pending on the quality of the acrylic used as well as the *Candida* species in question. Heat-cured acrylics in general tended to have significantly lesser number of yeast attached than cold-cured acrylics. Thus, heat-cured materials, such as Vertex, might be clinically the best choice. Moreover, candidal adhesion to denture acrylics is reduced to a great extent following pre-treatment of acrylic strips with nystatin (Ellepola and Samaranayake, 1998). Further, it has been documented that pre-treatment of BECs with nystatin resulted in reduction in candidal adhesion (Darwazeh et al., 1997). These studies indicate that pre-exposure of either the target surface or the yeast to nystatin results in reduced adhesion, which in clinical terms may prevent *Candida* adhesion and colonisation in the oral cavity.

CONCLUSION

From this review, it is evident that there are only a few *in vitro* studies on the adhesion of non-albicans *Candida* species with a limited number of isolates. As there are significant intra-species variations in *Candida* adhesion, it is important to evaluate a large number of isolates in order to elicit differences in relative adhesion among *Candida* species. It is not known to what extent the relative CSH contributes to colonization *in vivo* as other factors, such as saliva and the presence of bacteria on host surfaces, may confound this association. Further studies simulating the *in vivo* environment are required to confirm whether the observed phenomena operate intraorally.

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ОРАЛНА КАНДИДИЈАЗА — АДХЕЗИЈА NON-ALBICANS *CANDIDA* SPP.

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Резиме

Инфекција гљивом рода *Candida* представља проблем од све већег клиничког значаја. У последње две деценије, преваленција оралне гљивичне инфекције је енормно повећана, вероватно због повећања популације имунокомпетентних пацијената. Орална кандидијаза је опортунистичка инфекција примарно изазвана *C. albicans*. Међутим, у последњих неколико година уочено је да је ова инфекција много чешће изазвана врстама рода non-albicans *Candida. Candida* као коменсал чини део нормалне микрофлоре усне дупље. Познавање начина трансформације из коменсала у патогену форму, и како се она може спречити, је непрекидан изазов за клиничке лекаре. Адхезија гљиве рода *Candida* за површину слузокоже представља критичан, први корак за насељавање и настанак инфекције, као и у патогенези оралне кандидијазе. Акрилатне протезе, делујући као резервоари гљиве *Candida*, играју важну улогу у повећању ризика од насељавања усне дупље. Због тога је у овом раду приказан преглед литературе који се односи на адхезију оралних изолата non-albicans *Candida* spp. за епителне ћелије усне дупље и акрилатне протезе.

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THE IMPORTANCE OF GENUS *CANDIDA* IN HUMAN SAMPLES*

ABSTRACT: Microbiology is a rapidly changing field. As new researches and experiences broaden our knowledge, changes in the approach to diagnosis and therapy have become necessary and appropriate. Recommended dosage of drugs, method and duration of administration, as well as contraindications to use, evolve over time all drugs. Over the last 2 decades, *Candida* species have emerged as causes of substantial morbidity and mortality in hospitalized individuals. Isolation of *Candida* from blood or other sterile sites, excluding the urinary tract, defines invasive candidiasis. *Candida* species are currently the fourth most common cause of bloodstream infections (that is, candidemia) in U.S. hospitals and occur primarily in the intensive care unit (ICU), where candidemia is recognized in up to 1% of patients and where deep-seated *Candida* infections are recognized in an additional 1 to 2% of patients. Despite the introduction of newer anti-*Candida* agents, invasive candidiasis continues to have an attributable mortality rate of 40 to 49%; excess ICU and hospital stays of 12.7 days and 15.5 days, respectively, and increased care costs. Postmortem studies suggest that death rates related to invasive candidiasis might, in fact, be higher than those described because of undiagnosed and therefore untreated infection. The diagnosis of invasive candidiasis remains challenging for both clinicians and microbiologists. Reasons for missed diagnoses include nonspecific risk factors and clinical manifestations, low sensitivity of microbiological culture techniques, and unavailability of deep tissue cultures because of risks associated with the invasive procedures used to obtain them. Thus, a substantial proportion of invasive candidiasis in patients in the ICU is assumed to be undiagnosed and untreated. Yet even when invasive candidiasis is diagnosed, culture diagnosis delays treatment for 2 to 3 days, which contributes to mortality. Interventions that do not rely on a specific diagnosis and are implemented early in the course of *Candida* infection (that is, empirical therapy) or before *Candida* infection occurs (that is, prophylaxis) might improve patient survival and may be warranted. Selective and nonselective administration of anti-*Candida* prophylaxis is practiced in some ICUs. Several trials have tested this, but results were limited by low statistical power and choice of outcomes. Thus, the role of anti-*Candida* prophylaxis for patients in the ICU remains controversial. Initiating anti-*Candida* therapy for patients in the ICU who have suspected infection but have not responded to antibacterial therapy (empirical therapy) is practiced in some hospitals. This practice, however, remains a subject of

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considerable debate. These patients are perceived to be at higher risk from invasive candidiasis and therefore are likely to benefit from empirical therapy. Nonetheless, empirical anti-*Candida* therapies have not been evaluated in a randomized trial and would share shortcomings that are similar to those described for prophylactic strategies. Current treatment guidelines by the Infectious Diseases Society of America (IDSA) do not specify whether empirical anti-*Candida* therapy should be provided to immunocompetent patients. If such therapy is given, IDSA recommends that its use should be limited to patients with *Candida* colonization in multiple sites, patients with several other risk factors, and patients with no uncorrected causes of fever. Without data from clinical trials, determining an optimal anti-*Candida* strategy for patients in the ICU is challenging. Identifying such a strategy can help guide clinicians in choosing adequate therapy and may improve patient outcomes. In our study, we developed a decision analytic model to evaluate the cost-effectiveness of empirical anti-*Candida* therapy given to high-risk patients in the ICU, defined as those with altered temperature (fever or hypothermia) or unexplained hypotension despite 3 days of antibacterial therapy in the ICU.

KEY WORDS: *Candida*, species, human samples, candidiasis

INTRODUCTION

Background: *Candida* species are ubiquitous fungi and are the most common fungal pathogens that affect humans. The growing problem of mucosal and systemic candidiasis reflects the enormous increase in the pool of patients at risk and the increased opportunity that exists for *Candida* species to invade tissues normally resistant to invasion. *Candida* species are true opportunistic pathogens that exploit recent technological advances to gain access to the circulation and deep tissues (1).

The increased prevalence of local and systemic disease caused by *Candida* species has resulted in numerous new clinical syndromes, primarily dependent on the immune status of the host. *Candida* species produce a wide spectrum of diseases, ranging from superficial mucocutaneous disease to invasive illnesses, such as hepatosplenic candidiasis, *Candida* peritonitis, and systemic candidiasis. Management of serious and life-threatening invasive candidiasis remains severely hampered by delays in diagnosis and the lack of reliable diagnostic methods that allow detection of both fungemia and tissue invasion by *Candida* species.

Advances in medical technology, chemotherapeutics, cancer therapy, and organ transplantation have had a major impact on reducing the morbidity and mortality of life-threatening disease. Patients who are critically ill and in medical and surgical ICUs have been the prime targets for opportunistic nosocomial fungal infections, primarily due to *Candida* species. Studies suggest that the problem is not under control and, that it is, in fact, worsening. On a daily basis, virtually all physicians are confronted with a positive *Candida* isolate obtained from one or more various anatomical sites. High-risk areas for *Candida* infection include neonatal, pediatric, and adult ICUs, both medical and surgical. *Candida* infections can involve any anatomical structure (2).

Pathophysiology: *Candida* species are yeastlike fungi that can form true hyphae and pseudohyphae. For the most part, *Candida* species are confined to human and animal reservoirs. However, they are frequently recovered from the

hospital environment, including foods, counter tops, air-conditioning vents, floors, respirators, and medical personnel. They are also normal commensals of diseased skin and mucosal membranes of the GI, genitourinary, and respiratory tracts.

Candida species also contain their own set of well-recognized virulence factors. Although not well characterized, several virulence factors may contribute to their ability to cause infection. The main virulence factors are surface molecules that permit adherence of the organism to other structures (e.g., human cells, extracellular matrix, prosthetic devices), acid proteases, and the ability to convert to a hyphal form.

As with most fungal infections, host defects also play a significant role in the development of candidal infections. Numerous host defects are associated with candidal infections.

Host defence mechanisms against *Candida* infection and their associated defects that allow infection are as follows:

— Intact mucocutaneous barriers — Wounds, intravenous catheters, burns, ulcerations
— Phagocytic cells — Granulocytopenia
— Polymorphonuclear leukocytes — Chronic granulomatous disease
— Monocytic cells — Myeloperoxidase deficiency
— Complement — Hypocomplementemia
— Immunoglobulins — Hypogammaglobulinemia
— Cell-mediated immunity — Chronic mucocutaneous candidiasis, diabetes mellitus, cyclosporin A, corticosteroids, HIV infection
— Mucocutaneous protective bacterial florae — Broad-spectrum antibiotics

Risk factors associated with candidiasis include the following:

— Granulocytopenia
— Bone marrow transplantation
— Solid organ transplantation (liver, kidney)
— Parenteral hyperalimentation
— Hematologic malignancies
— Foley catheters
— Solid neoplasms
— Recent chemotherapy or radiation therapy
— Corticosteroids
— Broad-spectrum antibiotics
— Burns
— Prolonged hospitalization
— Severe trauma
— Recent bacterial infection
— Recent surgery
— GI tract surgery
— Central intravascular access devices

— Premature birth
— Hemodialysis

The first step in the development of a candidal infection is colonization of the mucocutaneous surfaces. The factors outlined above are all associated with increased colonization rates. The routes of candidal invasion are (1) disruption of a colonized surface (skin or mucosa), allowing the organisms access to the bloodstream, and (2) persorption via the GI wall, which may occur following massive colonization with large numbers of organisms that pass directly into the bloodstream.

Frequency

— **In the US:** *Candida* species are the most common cause of fungal infection affecting immunocompromised patients. Oropharyngeal colonization is found in 30—55% of healthy young adults, and *Candida* species may be detected in 40—65% of normal fecal florae.

Three of every 4 women have at least 1 bout of vulvovaginal candidiasis (VVC) during their lifetime.

In HIV-positive persons who are not receiving highly active antiretroviral therapy (HAART), more than 90% experience oropharyngeal candidiasis (OPC) and 10% have at least 1 episode of esophageal candidiasis.

In persons with systemic infections, *Candida* species are now the fourth most commonly isolated pathogens from blood cultures.

Clinical and autopsy studies have confirmed a considerable increase in the incidence of disseminated candidiasis, reflecting a parallel increase in the frequency of candidemia. This increase is multifactorial in origin and reflects increased recognition of the fungus, a growing population of patients at risk (i.e., patients undergoing complex surgical procedures, patients with indwelling vascular devices), and the improved survival of patients with underlying neoplasms or collagen-vascular disease and patients who are immunosuppressed (3,4).

— **Internationally**: Similar rates of mucocutaneous and systemic candidiasis have been observed worldwide. In fact, throughout the world, *Candida* species have replaced *Cryptococcus* species as the most common fungal pathogens affecting immunocompromised hosts.

Mortality/Morbidity

— Mucocutaneous candidiasis: Most candidal infections are mucocutaneous and, as such, do not cause mortality. However, in patients with advanced immunodeficiency due to HIV infection, these mucosal infections can become refractory to antifungal therapy and may lead to severe oropharyngeal and esophageal candidiasis that initiates a vicious cycle of poor oral intake, malnutrition, wasting, and early death.

— Candidemia and disseminated candidiasis: Mortality rates for these infections have not improved much over the past few years and remain in the range of 30—40%. Systemic candidiasis is the cause of more case fatalities than any other systemic mycosis. More than a decade ago, investigators reported the enormous economic impact of systemic candidiasis in hospitalized patients. Candidemia is associated with considerable prolongation of length of stay in the hospital (70 vs. 40 days in patients who are comparable, matched, and nonfungemic). Although mucocutaneous fungal infections, such as oral thrush and *Candida* esophagitis, are extremely common in patients with AIDS, candidemia and disseminated candidiasis are uncommon (5, 6, 7).

Sex: Colonization with *Candida* species occurs in equal numbers of males and females. However, in women, VVC is the second most common cause of vaginitis.

Age: Candidal colonization is at the highest levels during the age extremes in neonates and in people older than 65 years. In addition, mucocutaneous candidiasis is also more prevalent in neonates and older adults.

History: Infections due to *Candida* species can manifest in a wide spectrum of clinical syndromes as described below. The clinical presentation can vary depending on the type of infection and the degree of immunosuppression. Clinical syndromes associated with *Candida* infection are the following:

Cutaneous candidiasis syndromes

— Generalized cutaneous candidiasis: This is an unusual form of cutaneous candidiasis that manifests as a diffuse eruption over the trunk, thorax, and extremities. The patient has a history of generalized pruritus, with increased severity in the genitocrural folds, anal region, axillae, hands, and feet. Physical examination reveals a widespread rash that begins as individual vesicles that spread into large confluent areas.

— Intertrigo: The patient has a history of intertrigo affecting any site where the skin surfaces are in close proximity, providing a warm and moist environment. Pruritic red rash occurs. Physical examination reveals a rash that begins with vesiculopustules, which enlarge and rupture, causing maceration and fissuring. The area involved has a scalloped border with a white rim consisting of necrotic epidermis that surrounds the erythematous macerated base. Satellite lesions are frequently found and may coalesce and extend into larger lesions (8, 9).

— Metastatic skin lesions: Characteristic skin lesions occur in approximately 10% of patients with disseminated candidiasis and candidemia. The lesions may be numerous or few. Lesions are generally described as erythematous, firm, nontender macronodular lesions with discrete borders. Biopsy specimens of these lesions demonstrate yeast cells, hyphae, or pseudohyphae, and cultures are positive for *Candida* species in approximately 50% of the cases.

— *Candida* folliculitis: The infection is found predominantly in the hair follicles and, rarely, can become extensive.

— Paronychia and onychomycosis: Frequently, paronychia and onychomycosis are associated with immersion of the hands in water and with diabetes mellitus. The patient has a history of a painful and erythematous area around and underneath the nail and nail bed. Physical examination reveals an area of inflammation that becomes warm, glistening, tense, and erythematous and may extend extensively under the nail. It is associated with secondary nail thickening, ridging, discoloration, and occasional nail loss (10, 11).

Chronic mucocutaneous candidiasis

Chronic mucocutaneous candidiasis describes a group of *Candida* infections of the skin, hair, nails, and mucous membranes that tends to have a protracted and persistent course.

— History: Most infections begin in infancy or the first 2 decades of life; onset in people older than 30 years is rare.

- Most patients survive for prolonged periods and rarely experience disseminated fungal infections. The most common cause of death is bacterial sepsis.
- Chronic mucocutaneous candidiasis is frequently associated with endocrinopathies, such as the following:
  - Hypoparathyroidism
  - Addison disease
  - Hypothyroidism
  - Diabetes mellitus
  - Autoimmune antibodies to adrenal, thyroid, and gastric tissues (approximately 50%)
  - Thymomas
  - Dental dysplasia
  - Polyglandular autoimmune disease
  - Antibodies to melanin-producing cells

— Physical examination: Findings reveal disfiguring lesions of the face, scalp, hands, and nails. This is occasionally associated with oral thrush and vitiligo.

GI tract candidiasis

— Oropharyngeal candidiasis

- The patient has a history of HIV infection, denture wear, diabetes mellitus, or frequent use of broad-spectrum antibiotics or inhaled steroids. Patients may be asymptomatic, but variable symptoms may include the following:
  - Sore and painful mouth
  - Burning mouth or tongue

- Dysphagia
- Whitish, thick patches on the oral mucosa

- Physical examination reveals a diffuse erythema and white patches that appear on the surfaces of the buccal mucosa, throat, tongue, and gums. The following are the 5 types of OPC:

  - Membranous candidiasis: This is one of the most common types and is characterized by creamy-white curdlike patches on the mucosal surfaces.
  - Erythematous candidiasis: This is associated with an erythematous patch on the hard and soft palates.
  - Chronic atrophic candidiasis (denture stomatitis): This type is also thought to be one of the most common forms of the disease. The presenting signs and symptoms include chronic erythema and edema of the portion of the palate that comes into contact with dentures.
  - Angular cheilitis: An inflammatory reaction, this type is characterized by soreness, erythema, and fissuring at the corners of the mouth.
  - Mixed: A combination of any of the above types is possible.

— Esophageal candidiasis

- The patient's history usually includes chemotherapy, the use of broad-spectrum antibiotics or inhaled steroids, or the presence of HIV infection or hematologic or solid organ malignancy. Patients may be asymptomatic, but variable symptoms may include the following:

  - No oral disease (> 50% of patients)
  - Dysphagia
  - Odynophagia
  - Retrosternal pain
  - Epigastric pain
  - Nausea and vomiting

- Upon physical examination, oral candidiasis is nearly always present.

— Nonesophageal GI candidiasis

- Most commonly, the patient's history includes an association with neoplastic disease of the GI tract. The stomach is found to be the second most commonly infected site after the esophagus. With less frequency, patients may have chronic gastric ulcerations, gastric perforations, or malignant gastric ulcers with concomitant candidal infection. The third most common site of infection (20%) is the small bowel. The frequency of candidal infection in the small bowel is the same as in the large bowel. Approximately 15% of patients develop systemic candidiasis.

- Physical examination findings are variable and depend on the site of infection. The diagnosis, however, cannot be made solely on culture results because approximately 20—25% of the population is colonized by *Candida*. The following symptoms may be present:
  - Epigastric pain
  - Nausea and vomiting
  - Abdominal pain
  - Fever and chills
  - Occasionally, abdominal mass

Respiratory tract candidiasis

The respiratory tract is frequently colonized with *Candida* species, especially in hospitalized patients. In ambulatory patients, 20—25% of the population is colonized by *Candida* species.

— Laryngeal candidiasis: This is very unusual but may be a source for disseminated candidiasis. Laryngeal candidiasis is primarily observed in patients with hematologic malignancies. The patient may have a sore throat and hoarseness. Physical examination findings are generally unremarkable, and the diagnosis is made by direct or indirect laryngoscopy.

— *Candida* tracheobronchitis: This is a rare form of candidiasis. Most patients with *Candida* tracheobronchitis are seropositive for HIV, or are severely immunocompromised, reporting fever, productive cough, and shortness of breath. Physical examination reveals dyspnea and scattered rhonchi. The diagnosis is generally made after bronchoscopy.

— *Candida* pneumonia: It does not exist alone and occurs only rarely as a part of disseminated candidiasis. The most common form is multiple abscesses due to hematogenous dissemination of *Candida* species. The high degree of colonization and isolation of *Candida* species from the respiratory tract makes diagnosing this entity difficult. The patient's history reveals similar risk factors for disseminated candidiasis, and patients report shortness of breath, cough, and respiratory distress. Physical examination reveals fever, dyspnea, and variable breath sounds, from clear, rhonchi to scattered rales.

Genitourinary tract candidiasis

— Vulvovaginal candidiasis: This is the second most common cause of vaginitis. The patient's history includes vulvar pruritus, vaginal discharge, dysuria, and dyspareunia. Approximately 10% of women experience repeated attacks of VVC without precipitating risk factors. Physical examination findings include a vagina and labia that are usually erythematous, a thick curdlike discharge, and a normal cervix upon speculum examination.

— *Candida* balanitis: Patients report itchiness of the penis. Lesions and whitish patches are present. *Candida* balanitis is acquired through sexual intercourse with a partner who has VVC. Physical examination reveals vesicles on

the penis that develop later into patches resembling thrush. The rash may spread to the thighs, gluteal folds, buttocks, and scrotum.

— *Candida* cystitis: Many patients frequently are asymptomatic. However, bladder invasion may result in frequency, urgency, dysuria, hematuria, and suprapubic pain. *Candida* cystitis may or may not be associated with the use of a Foley catheter. Physical examination may reveal suprapubic pain; otherwise, examination findings are unremarkable.

— Asymptomatic candiduria: Most catheterized patients with persistent candiduria are asymptomatic, similar to noncatheterized patients. Most patients with candiduria have easily identifiable risk factors for *Candida* colonization. Thus, the distinction between invasive disease and colonization cannot be made solely on culture results because approximately 5—10% of all urine cultures are positive for *Candida*.

— Ascending pyelonephritis: The use of stents and indwelling devices, along with the presence of diabetes, is the major risk factor predisposing patients to ascending infection. The patient frequently has a history of flank pain, abdominal cramps, nausea, vomiting, fever, chills, and hematuria, Physical examination reveals abdominal pain, costovertebral-angle tenderness, and fever.

— Fungal balls: This is due to the accumulation of fungal material in the renal pelvis. The condition may produce intermittent urinary tract obstruction with subsequent anuria and ensuing renal insufficiency (12, 13).

— Candidemia

- *Candida* species currently are the fourth most commonly isolated organism in blood cultures, and *Candida* infection generally is considered a nosocomially acquired infection. The patient's history commonly reveals the following:
  - Several days of fever that is unresponsive to broad-spectrum antimicrobials; often the only marker of infection
  - Prolonged intravenous catheterization
  - A history of several key risk factors (see http://www.emedicine.com/med/topic264.htm Pathophysiology)
  - Possibly associated with multiorgan infection.
- Physical examination is remarkable for the following:
  - Fever
  - Macronodular skin lesions (approximately 10%)
  - Candidal endophthalmitis (approximately 10—28%)
  - Occasionally, septic shock (hypotension, tachycardia, tachypnea).
- Other causes of candidemia without invasive disease include the following:
  - Intravascular catheter-related candidiasis: This entity usually responds promptly to catheter removal and antifungal treatment.
  - Suppurative thrombophlebitis: For the most part, this is observed secondary to prolonged central venous catheterization. Suppura-

tive thrombophlebitis manifests as fever and candidemia, which persist despite antifungal therapy and catheter removal. Sepsis also may be present.

- Endocarditis: The frequency of endocarditis has increased in the past few years. Endocarditis is the most common cause of fungal endocarditis and is primarily due to *Candida albicans* (> 60% of cases). The most common valves involved are the aortic and mitral. The 2 different forms of endocarditis are exogenous, which is secondary to direct infection during surgery, and endogenous, which is due to secondary spread during candidemia and disseminated candidiasis. Endocarditis is frequently associated with 4 main risk factors. These are (1) intravenous heroin use, which is frequently associated with infection due to *Candida* parapsilosis; (2) chemotherapy; (3) prosthetic valves (approximately 50%); and (4) prolonged use of central venous catheters.

— Disseminated candidiasis: This is frequently associated with multiple deep organ infections or may involve single organ infection. Unfortunately, patients with disseminated candidiasis, as many as 40—60% of them, may have blood culture results negative for *Candida* species. The history of a patient with presumptive disseminated candidiasis reveals a fever unresponsive to broad-spectrum antimicrobials and negative results from blood culture. Physical examination reveals fever (which may be the only symptom) with an unknown source and sepsis and septic shock.

— *Candida* endophthalmitis: The 2 forms of *Candida* endophthalmitis are the exogenous and the endogenous form. Exogenous endophthalmitis is associated with either accidental or iatrogenic (postoperative) injury of the eye and inoculation of the organism from the environment. Endogenous endophthalmitis results from hematogenous seeding of the eye. It is found in 10—28% of the patients with candidemia. The use of hematogenous candidal endophthalmitis as a marker of widespread disseminated candidiasis is important.

- The patient's history reveals a broad range of manifestations.
  - Eye injury
  - Ophthalmic surgery
  - Underlying risk factors for candidemia
  - Asymptomatic and detected upon physical examination
  - Ocular pain
  - Photophobia
  - Scotomas
  - Floaters
- Physical examination reveals fever.
- Upon funduscopic examination, early lesions are the size of a pinhead, are off-white in color, and are found in the posterior vitreous with distinct margins and minimal vitreous haze. Classic lesions are large and off-white, similar to a cotton-ball, with indistinct borders covered by an underlying haze. Lesions are 3-dimensional and ext-

end into the vitreous off the chorioretinal surface. They may be single or multiple.

— Renal candidiasis

- This is most frequently a consequence of candidemia and disseminated candidiasis. Patient's history includes fever that is unresponsive to broad-spectrum antimicrobials. Frequently, patients are asymptomatic and lack symptoms referable to the kidney.
- Physical examination is generally unremarkable, and renal candidiasis is diagnosed after urinalysis and renal biopsy. Otherwise, this condition is commonly diagnosed at autopsy.
- Physical examination reveals the following:
  - Fever
  - Nuchal rigidity
  - Confusion
  - Coma

— *Candida* arthritis, osteomyelitis, costochondritis, and myositis

- In the past, musculoskeletal infections were rare; currently, they are more common, due to the increased frequency of candidemia and disseminated candidiasis. The most common sites of involvement are the knee and vertebral column. The pattern of involvement is similar to the pattern observed in bacterial infections. The infection may be exogenous or endogenous. The exogenous infection is frequently due to direct inoculation of the organisms, such as postoperative infection or trauma. Affected sites include the following:
  - Ribs and leg bones (< 20 years)
  - Vertebral column and paraspinal abscess (adulthood)
  - Flat bones (any age group)
  - Sternum — Generally observed postoperatively after cardiac surgery
- The patient frequently is asymptomatic, and the patient's history reveals underlying risk factors of disseminated candidiasis and localized pain over the affected site. The physical examination is frequently unremarkable; otherwise, it may reveal tenderness over the involved area, erythema, and bone deformity, occasionally with a draining sinus.
  - Arthritis: Generally, arthritis is a complication of disseminated candidiasis, but it may be caused by trauma or direct inoculation due to surgery or steroid injections. Most cases are acute and begin as a suppurative synovitis. A high percentage of cases progress to osteomyelitis. In addition, developing *Candida* arthritis after joint replacement is not uncommon.
  - Osteomyelitis: The 2 forms of osteomyelitis are exogenous infection and endogenous infection. The exogenous infection is frequently due to either direct inoculation of the organisms, such as

through postoperative infection, trauma, or steroid injections. The endogenous form of osteomyelitis is generally a complication of disseminated candidiasis. Most cases, due to hematogenous seeding, infect the vertebral disks and progress to diskitis with extension into the vertebrae from contiguous spread. Other bones affected include the wrist, femur, scapula, and proximal humerus.

- Costochondritis: This is rare and usually has 2 forms. Costochondritis usually results from either hematogenous spread or direct inoculation during surgery (median sternotomy). Frequently, costochondritis is associated with localized pain over the involved area.
- Myositis: This occurs infrequently, and an association with disseminated candidiasis is common. Most patients are neutropenic. People with myositis have a history of muscular pain.

— Myocarditis-pericarditis: This is due to hematogenous spread in association with disseminated disease and is rarely due to direct extension from the sternum or esophagus. Myocarditis-pericarditis occurs as diffuse abscesses scattered throughout the myocardium with normal cardiac tissue. In persons with disseminated candidiasis, the rate has been documented to be as high as 50%. The patient's history reveals serious complications in 10—20% of the cases without valve disease, fever and chills. Physical examination reveals fever, hypotension, shock, tachycardia, and new murmurs or rubs (changes in previously detected murmurs).

— *Candida* peritonitis

- The patient's history frequently reveals an association with GI tract surgery, viscous perforation, or peritoneal dialysis. *Candida* peritonitis tends to remain localized, and only in 15% of the cases does the infection disseminate into the blood stream. The range of manifestations is broad and includes fever and chills, abdominal pain and cramping, nausea and vomiting, and constipation.
- Physical examination is significant for the following:
  - Fever
  - Abdominal distention
  - Abdominal pain
  - Absent bowel sounds
  - Rebound tenderness
  - Localized mass

— *Candida* splenic abscess and hypersplenism: Both are manifestations of disseminated candidiasis and are usually simultaneously associated with liver involvement. Manifestations of hypersplenism are common (see http://www.emedicine.com/med/topic264.htm Hepatosplenic candidiasis).

— *Candida* cholecystitis: This is rare and generally associated with bacterial cholangitis and ascending cholangitis. Most commonly, *Candida* cholecystitis is diagnosed at the time of surgery when a culture is obtained.

— The medically significant *Candida* species include the following:

- *C. albicans*, the most common species identified (50—60%)
- *C. glabrata* (15—20%)
- *C. parapsilosis* (10—20%)
- *C. tropicalis* (6—12%)
- *C. krusei* (1—3%)
- *C. kefyr* (< 5%)
- *C. guilliermondi* (< 5%)
- *C. lusitaniae* (< 5%)
- *C. dubliniensis*, primarily recovered from patients who are positive for HIV.

— *C. glabrata* and *C. albicans* account for approximately 70—80% of yeast isolated from patients with invasive candidiasis. *C. glabrata* has recently become important because of its increasing incidence worldwide, and it is intrinsically less susceptible to azoles and amphotericin B.

— *C. krusei* is important because of its intrinsic resistance to ketoconazole and fluconazole (Diflucan). Additionally, it is also less susceptible to all other antifungals, including itraconazole (Sporanox) and amphotericin B.

— Another important *Candida* species is *C. lusitaniae*; although not as common as some *Candida* species, it is of clinical significance because it is frequently resistant to amphotericin B, although it remains susceptible to azoles and echinocandins.

— *C. parapsilosis* is an important species to consider in hospitalized patients with vascular catheters.

— *C. tropicalis* has been considered an important cause of candidemia in patients with cancer (leukemia), and in those who have undergone bone marrow transplantation.

Lab Studies

— Unfortunately, findings from the laboratory studies are often nonspecific. Clinicians are required to act definitively and early, based on a high index of suspicion. In the past, many patients with life-threatening candidiasis died without receiving antifungal therapy. Patients who remain febrile despite broad-spectrum antibiotic therapy, with either persistent neutropenia or other risk factors and persistent leukocytosis, should be suspected of having systemic candidiasis. To be effective, therapy should be provided early and empirically in such patients.

— Cultures of nonsterile sites, although not useful for establishing a diagnosis, may demonstrate high degrees of candidal colonization. Positive culture results from sterile sites should be considered significant and as an evidence of infection.

— Mucocutaneous candidiasis

- Wet mount, scrapings or smears obtained from skin, nails, oral mucosa, or vaginal mucosa are examined under the microscope for hyphae, pseudohyphae, or budding yeast cells.
- With a potassium hydroxide smear, the Gram stain methylene blue is useful to demonstrate fungal cells, directly.
- Cultures of affected nails are helpful to diagnose onychomycosis versus noninfectious causes.

— Candidemia and disseminated candidiasis

- Blood cultures are helpful but are positive in only 50—60% of the cases of disseminated disease.
- Urinalysis may be helpful, and results may be indicative of either colonization or renal candidiasis.
- The serum 1—3 D-glucan detection assay (Glucatell, Fungitell) is a nonculture test, which was approved for use in the United States in May 2004. This assay measures the level of beta-glucan (a fungal cell wall component). In a large multicenter study, the assay had a high specificity and positive predictive value with highly reproducible results.
- Cultures of nonsterile sites, although not useful for establishing a diagnosis, may be useful for initiating antifungal therapy in patients with fever that is unresponsive to broad-spectrum antimicrobials. Therefore, appropriate interpretation is required. Positive results from blood cultures and cultures from other sterile sites imply the presence of invasive disease. Always consider positive results from these sites to be significant and to be an evidence of infection.
- GI, respiratory, and urinary tract culture results positive for *Candida* may not represent invasive disease. However, consider the GI, respiratory, and urinary tract sites of colonization.

— Cutaneous candidiasis: Use a wet mount. Scrapings or smears obtained from skin or nails are examined under the microscope for hyphae, pseudohyphae, or budding yeast cells. Potassium hydroxide smears are also useful.

— Genitourinary candidiasis: Perform a urinalysis. Evidence of WBCs, RBCs, protein, and yeast cells can be found. Additionally, urine fungal cultures are useful.

— Respiratory tract candidiasis

- Sputum Gram stain demonstrates WBCs and yeast cells.
- Sputum culture demonstrates *Candida* species.
- Lung biopsy is mandatory to establish definitively the diagnosis of respiratory tract candidiasis, because the respiratory tract is frequently colonized with yeast.

— GI candidiasis: Endoscopy, with or without biopsy is necessary to establish the diagnosis.

— Focal hepatosplenic candidiasis: Elevation of the serum alkaline phosphatase level is common.

— Species identification

- *C. albicans, C. dubliniensis*, and *C. stellatoidea* can be identified morphologically by germ-tube formation (hyphae are produced from yeast cells after 2—3 h of incubation) or biochemical assays.
- CHROMagar *Candida* allows presumptive identification of several *Candida* species by using color reactions in specialized media that demonstrate different colony colors, depending on the species of *Candida.*
- API 20C and API 32C are biochemical assays that allow the identification of different *Candida* species with more precision. These assays evaluate the assimilation of a number of carbon substrates and generate profiles used in the identification of different fungal species.

— Antifungal susceptibility testing

- *In vitro* susceptibility testing for *Candida* species is now standardized, using the National Committee for Clinical Laboratory Standards (NCCLS) microbroth dilution methodology (NCCLS M27-A2).
- Although not used as a standard of care, this method may be helpful in guiding difficult therapeutic decisions. Most of the difficult decisions are observed in antifungal, refractory, oral, or esophageal candidiasis in patients with advanced HIV disease.

— Nonculture *Candida* detection assays

- The *Candida* mannan assay has a sensitivity of 31—90% (less for non-*albicans Candida* species).
- The *Candida* heat labile antigen assay has a sensitivity of 10—71%.
- The D-arabinitol assay has a sensitivity of 50% but is not useful for infection with *C. krusei* or *C. glabrata*.
- The enolase assay has a sensitivity of 55—75%, which improves with serial testing.
- The 1—3 beta-D-glucan assay is an amebocyte lysis assay with a sensitivity of 75—100% and a specificity of 88—100% (broad--spectrum assay that detects *Aspergillus, Candida, Fusarium, Acremonium*, and *Saccharomyces* species). Beta-D-glucan is a component of the cell wall of a wide variety of fungi and can be detected by its ability to activate factor G of the horseshoe crab coagulation cascade. The Fungitell assay is used in the evaluation of invasive fungal infections caused by the species mentioned above to guide diagnosis. It does not detect infections caused by *Cryptococcus neoformans* and Zygomycetes.

— Molecular assays such as polymerase chain reaction tests and DNA probes are still under development and in the early reserach stage.

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ЗНАЧАЈ РОДА *CANDIDA* ЗА ЉУДСКЕ УЗОРКЕ

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Резиме

Микробиологија представља науку која се развија прогресивно. Најновија истраживања показују посебне измене на пољу дијагнозе и одговарајуће терапије. Током последње две декаде, кандида специјес добиле су на значају као узрочници морбидитета и морталитета код хоспитализованих пацијената. Изолација кандида из крви или других стерилних подручја искључујући уринарни тракт, потврђује инвазивност кандида. Кандида врсте налазе се на четвртом месту најчешћих узрочника који изазивају инфекције преко крви (кандидемија) у САД, а нарочито на одељењима интензивне неге где се код 1% пацијената ради о дубоким инфекцијама узрокованим кандида врстама. Упркос увођењу новијих антикандида агенаса, инвазивна кандидијаза остаје и даље изазов за клиничаре и микробиологе. Разлози за пропусте у дијагностици укључују неспецифичне факторе ризика и клиничке манифестације, слабу осетљивост техника култивације кандида и примену инвазивних метода за узорковање болесничког материјала у случају дубоке кандидијазе. Чак и када се инвазивна кандидијаза дијагностикује, култивација кандида траје 2—3 дана, што доприноси порасту морталитета. Превентивно давање (емпиријска терапија) антикандида агенаса може побољшати преживљавање пацијената. Селективно и неселективно давање антикандида агенаса у виду профилаксе примењује се на неким одељењима интензивне неге. Остаје контроверзна улога антикандида профилаксе код таквих пацијената, с обзиром на њихов одговор на антимикробну терапију. Водич за актуелни третман кандидијазе Удружења за инфективне болести САД (ИДСА) није посебно дефинисао да ли емпиријска антикандида терапија треба да се примени код имунокомпетентних пацијента. Посебно је тешко применити оптималну антикандида стратегију код хоспитализованих пацијената на одељењима интензивне неге.

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THE PRESENCE OF UNDESIRABLE MOULD SPECIES ON THE SURFACE OF DRY SAUSAGES*

ABSTRACT: Transition from manufacture to the industrial way of meat production and processing, as well as contemporary concept of food quality and safety, have led to the application of starter cultures. Their application leads towards the streamlining of the production process in the desired direction, quality improvement and its harmonization, and thereby to its standardization. Application of moulds in the meat industry is based on positive effects of their proteolytic and lipolytic egzoenzymes which, as a consequence, leads to the creation of characteristic sensory properties ("flavor") of fermented products. *Penicillium nalgiovense* is a typical representative of moulds used in the production of fermented sausages-salamis from our region.

Samples of "zimska salama" (dry sausage), produced with *Penicillium nalgiovense*, were evaluated as hygienically unacceptable. Their sensory properties changed due to contamination of this mould during the ripening process. Micological analysis discovered the presence of *Penicillium aurantiogriseum*, which is a frequent mould contaminant in the meat industry. At the same time, thin layer chromatography revealed no possibility of metabolic activity of this mould in the creation of mycotoxins. However, the presence of this mould on the surface of "zimska salama" is considered as undesirable due to formation of "off flavor" in products. Such product is considered as hygienically unacceptable and cannot be used for the human consumption.

KEYWORDS: dry sausage, mould, mycotoxin, off-flavor, *Penicillium nalgiovense*, *P. aurantiogriseum*

INTRODUCTION

Initial usage of moulds in the production of fermented sausages is connected with Italy and the year 1730, while in Hungary, their application was first introduced by two Italian butchers in 1835 (Leistner, 1986). Today, the application of moulds in meat and dairy industry is common in most of the

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European countries. Meat industry in southern-European countries, especially in Italy, Spain, France, Hungary and South Germany, uses positive properties of certain mould species during the fermentation of some fermented salamis (S u n e s e n & S t a h n k e, 2003). Their controlled application is ensured by the application of starter cultures.

Positive effects of moulds, such as the creation of desirable sensory characteristics of fermented sausages, so called "flavor", are the result of their influence on the proteolytic processes, lactate oxidation, amino acid degradation, lipolysis, β-oxidation, occurring during the maturing process (G r a t i a at al., 1986; L e i s t n e r, 1984; L ü c k e, 1997; C o o k, 1995; L ü c k e, 1998). No less significance is attributed to the protective effect of moulds, colonized on the surface of the sausage, against other undesirable species of yeasts, moulds and bacteria (L u c k e & H e c h e l m a n n, 1987). The positive effect on the colour stabilization process, as well as the process of postponing the product's rancidity, was also described. These effects are the result of emphasized enzymatic — catalase activity of added moulds, oxygen consumption and protection from light (B a c u s, 1986; L u c k e & H e c h e l m a n n, 1987). Furthermore, white and grayish mycelia of colonized moulds on the surface of the sausage, decreases the possibility of additional drying and the occurrence of the so called "gray edge" (L ü c k e, 1997), simultaneously forming smooth surface and uniform appearance of the product (G r a z i a, 1986; S u n e s e n & S t a h n k e, 2003).

Besides the fact that many of the meat industries are using native mould cultures for the production of certain fermented sausages, the usage of starter cultures with already determined desired functional properties, is more and more in practice. This approach eliminates the risk of obtaining a product with undesirable sensory properties, as well as poisoning caused by mycotoxins (C o o k, 1995).

As a result of inadequate, unprofessional application of moulds in the meat industry, the economic losses can be extremely high, especially if the mistakes are connected to the maturing process involving large part of production lot.

Penicillium nalgiovense (Figure 1) represents the typical mould used in the European meat industry (L u d e m a n n, 2004). Numerous researches dedicated to the examination of the *P. nalgiovense* application in the production of raw sausages, have shown its exquisite potential and superiority compared to other mould species from genus *Penicillium*. Today, many authorities in this area approve its application in commercial starter cultures (S u n e s e n & S t a h n k e, 2003; G a r c i a et al., 2001).

In our country, *P. nalgiovense* is used for the production of "zimska salama" (R a d e t i ć, 1997). The revitalized culture, in the concentration of cca 10^6 cfu/ml water, is sprayed on the surface of the sausages during certain maturing phases. Guided by the principles of good laboratory practice, and eliminating the risk of a consequent contamination, it is also possible to use broth mould culture made at meat industry plant laboratories.

MATERIALS AND METHODS

Due to the presence of the spoilage elements three months after the production the samples of "zimska salama" were subjected to the sensory analysis, as well as detailed mycological analysis, aiming at determination of colonized mould species, occurring on the surface of the product. At the same time, myco-toxicological analysis was conducted to establish mycotoxin production ability of the isolated mould.

Mycological analysis

Mould isolation was conducted on Sabouraud — 4 Maltose Agar, (Merck) (European Pharmacopeia II) having the following composition: peptone 10,0 g, D(+) glucose 40,0 g, agar-agar 15,0 g. Identification of the mould of different age (7, 14, 21. and 28. days) isolated and cultivated under laboratory conditions, was conducted according to the key described by R. A. Samson et al. (2004).

Myco-toxicological analysis

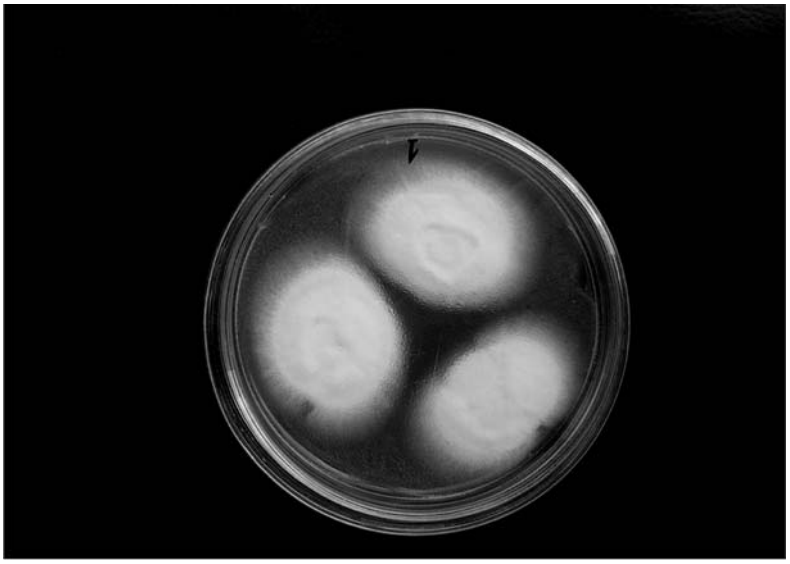
The isolated mould culture, during its growth under laboratory conditions (temperature 4° and 25°C, growth period 14 and 21 days), was examined with the aim of determining the potential production of extra cellular metabolites — mycotoxines. Determination of ability for mycotoxin synthesis was done by thin layer chromatography, as described by Giemeno et. al. (1983). Toxin identification was achieved by various developing solvents, spray reagents, and chemical reactions, and then quantified. The minimum detectable concentration of the examined mycotoxin (ochratoxin A) was 10 μg/kg.

The obtained results were evaluated according to the enforced regulative of our country (The Official Gazette of FRY no. 5/92).

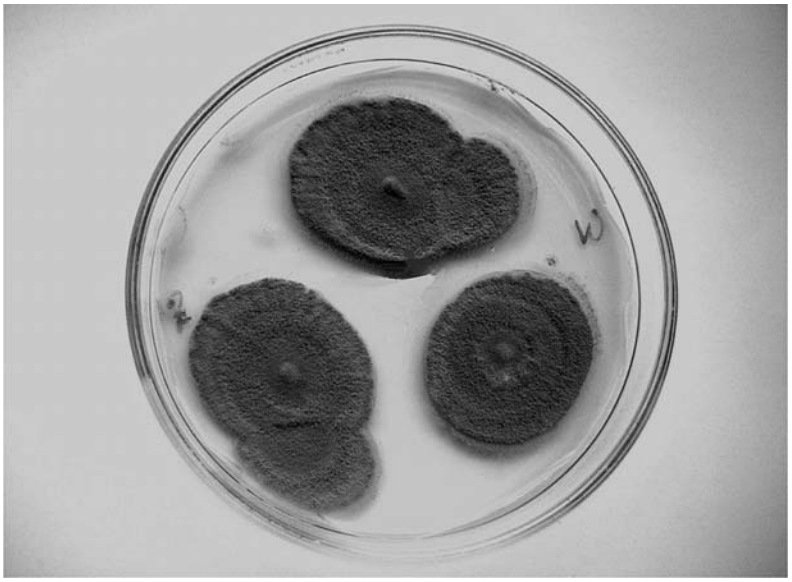
RESULTS AND DISCUSSION

The isolated mould culture, *P. aurantiogriseum* (synonym *P. verrucosum* var. *cyclopium*) is a frequent contaminant of the *P. nalgiovense* starter culture. The isolated mould culture (Figure 2) plays a significant role in contamination of fermented meat products (Horvat-Skenderović & Škrinjar, 1990). Source of contamination can be attributed mostly to the disrespect of basic principles of good hygienic practice, souch as inadequate plant hygiene, that is, hygiene of the working plateaus in direct contact with raw materials and end-products, as well as air contamination and, of course, human factor that can be of decisive influence (Škrinjar & Dimić, 1996). Of equal influence are the mycological profiles of raw materials, additives and spices (especially natural, thermally untreated), that can be significant sources of moulds (Horvat-Skenderović, 1989).

P. aurantiogriseum, according to the literature data is potentially toxic mould that can produce neurotoxic — verrucosidin (S c h e u e r, 1995), or nephrotoxic, carcinogen and immunotoxic — ochratoxin A (P e s t k a, 1995). The obtained results showed that the isolated and examined strain of *P. aurantiogriseum*, did not show the ability for mycotoxin-ochratoxin production. This



Slika br. 1 *Penicillium nalgiovense*



Slika br. 2 *Penicillium aurantiogriseum*

result was quite convenient from the consumers' point of view since this product could have come to the final user-human.

The sensory results implied that the samples of "zimska salama" were hygienically unacceptable, and due to the changed sensory properties, could not be used for human consumption (The Off. G. FRY no. 53/91 and annexes — The Off. G. FRY no. 24/94 and 28/96). Visible changes were especially emphasized on the surface of the casing and in the outer part of the filling, right beneath the casing. At these spots, the presence of moulds was recorded. Moulds were unevenly growing on the surface of salami, creating grayish layers in the form of stamps of different sizes. The aroma was adverse with expressed admixture note of mould, bitter and hot.

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Pravilnik o količinama pesticida, metala, metaloida i drugih otrovnih supstancija, hemioterapeutika, anabolika i drugih supstancija koje se mogu nalaziti u namirnicama (Sl. list SRJ br. 5/92).

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Zakon o zdravstvenoj ispravnosti životnih namirnica i predmeta opšte upotrebe (Sl. list SFRJ br. 53/91, 24/94, 28/96).

ПРИСУСТВО НЕПОЖЕЉНИХ ВРСТА ПЛЕСНИ НА ПОВРШИНИ СИРОВИХ КОБАСИЦА

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Резиме

Прелазак са занатске производње и прераде меса на индустријски начин, као и савремени концепт обезбеђења квалитета и безбедности хране, условили су употребу стартер-култура. Њихова примена доводи до усмеравања процеса зрења у жељеном правцу, побољшавању и уједначавању квалитета а тиме и стандардизацији производње. Истовремено, постиже се хигијенска сигурност у току производње као и добијање здравствено-безбедног производа. Употреба плесни у индустрији меса заснива се на позитивним ефектима њихових протеолитичких и липолитичких егзоензима, што последично доводи до настанка карактеристичних сензорских својстава (flavour) сирових производа. Незаобилазан је и заштитни ефекат, као и утицај на стабилизацију боје и успоравање настанка ранцидитета производа. Док је употреба бактерија и квасаца везана за деловање у унутрашњости надева кобасице, дотле је примена селекционисаних плесни везана за

површинску „контаминацију". Карактеристичан изглед (сиво-бела површина), типична арома производа као и заштитни ефекат, које остварују селекционисане и намерно додате, по површини, одређене врсте нетоксигених плесни, представљају основе њихове примене у индустрији меса.

Penicillium nalgiovense представља типичну врсту плесни која се користи на нашим просторима у производњи ферментисаних кобасица — салама.

У овом раду анализирани су узорци „зимске саламе", у чијој производњи је била употребљена култура *Penicillium nalgiovense* и који су органолептичком анализом оцењени као хигијенски неисправни. Узорци су имали измењена сензорска својства, настала као резултат процесне или постпроцесне контаминације у току процеса производње или, пак, неадекватног или неусловног складиштења. Миколошком анализом утврђено је присуство *Penicillium aurantiogriseum*, који је у литератури и пракси познат као чест контаминент плесни. Истовремено, методом танкослојне хроматографије, у лабораторијским условима гајења, није утврђена могућност њене метаболичке активности у правцу стварања микотоксина. Међутим, присуство ове врсте плесни, на површини „зимске саламе", сматра се непожељним, због настанка „of flavour-a" производа. Такав производ је хигијенски неисправан и неупотребљив за људску исхрану.

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EFFECTS OF MOULDS ON THE SAFETY AND PROCESSING QUALITY OF *TRITICUM AESTIVUM**

ABSTRACT: Wheat and wheat products are frequently subjected to mould infestations. Many of them are potential producers of various mycotoxins. Some of the consequences, due to the infestations by genus *Fusarium* and *Alternaria*, are mostly: yield loss, decrease of biological and technological quality, and unacceptable quality of infected kernels for the production and processing into human food because of the possible presence of mycotoxins.

It is unknown whether and how the contaminated grains are distributed during milling into various flour streams and finished products. Wholegrain flours and related products contain all anatomic parts of kernels, including mycotoxins. It is a known fact that mycotoxins are resistant to thermal degradation, so they do not loose their toxicity during processing. Moulds from genus *Fusarium* spp. and *Alternaria* spp. synthesize mycotoxins, mostly zearalenon and ochratoxin A.

The aim of the investigation was to examine mould contamination of wheat grain, as well as to identify the isolated species, especially those capable of producing toxins, and to determine their impact on technological quality, safety and sanitary condition of wheat.

Six varieties of wheat, contaminated with moulds, were investigated. Each sample was separated manually into four fractions: sound kernels, black germ kernels, kernels infected slightly and those infected severely with *Fusarium* spp.

KEY WORDS: moulds, mycotoxins, ochratoxin, technological quality, wheat, zearalenon

INTRODUCTION

Agricultural production is a complex processing cycle susceptible to contamination with field moulds and/or toxic metabolites, in all phases of production, transport, storage, and processing. The researches have been conducted to emphasize the necessary changes needed in comprehending that wheat is

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staple food and the most important chain in the biological cycle of the migration of hazardous contaminants, posing serious health concerns to humans.

Cereals, especially wheat, represent a strategic raw material from the aspect of human nutrition. However, cereals and cereal products can be contaminated with moulds in any phase of a processing cycle: in fields, during harvest, storage, processing, transport, and over a period between production and consumation. Many of the moulds are potential producers of mycotoxins (Šarić et al., 1973; Šarić et al., 2004).

The aim of the investigation was to examine mould contamination of wheat grain and wheat flour, as well as to identify the isolated species, especially those capable of producing toxins, and to determine their impact on technological quality, safety and sanitary condition of wheat.

MATERIALS AND METHODS

The paper presents results of investigation of 6 wheat varieties infested severely with *Fusarium* spp. and *Alternaria* spp. Kernels were separated on the basis of sensory properties, mycological and mycotoxical tests.

The contaminated kernels were separated into three fractions:

— **Black germ fraction** (Figure 1) comprises kernels with altered colour of outer layers, mostly around germ and crease of the wheat kernel. The colour changes are caused by different mould species or, so called, "black moulds", mostly belonging to the genera *Alternaria* and *Helminothosporum* (Šarić et al., 1973; Šarić et al., 2004; Šarić et al., 2004a; Šarić et al., 1980). The content of these kernels is not considered impure according to JUS E.B1.200 standard (1992).

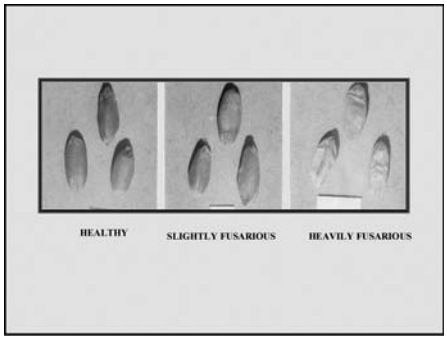


Fig. 1 — Kernels infested with moulds from genus *Fusarium*

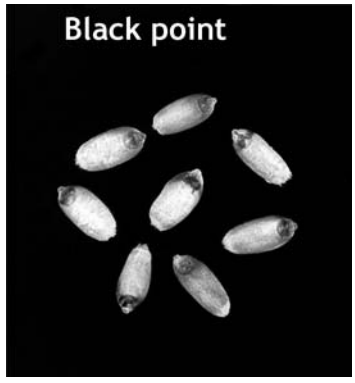


Fig. 2 — Kernels with blask germ

— **Fraction of kernels moderately contaminated with** *Fusarium* spp. consists of less shrivelled kernels with slightly expressed white or pink layers on the surface. These kernels are mostly contaminated with *Fusarium* spp. during waxy or full maturity of the wheat. It is not possible to separate this frac-

tion during milling cleaning so it enters flour. The highest contamination is usually observed in the aleuronic layer (Šarić et al., 1973; Šarić et al., 2004; Šarić et al., 2004a; Šarić et al., 1980).

— **Fraction of kernels severely contaminated with** *Fusarium* spp. (Figure 2) includes shrunk, and scabby kernels, coloured in white or light red. Such kernels are consequence of mould infestation in early stages of wheat maturity. During milling cleaning, these kernels are only partially removed, and mostly enter further processing phases (Šarić et al., 1973; Šarić et al., 2004; Šarić et al., 2004a; Šarić et al., 1980).

— Control sample consisted of **fraction of sound wheat kernels** from the corresponding wheat sample.

At collection terminals, harvested wheat usually contains all of the described fractions. Wheat bulk is sampled, evaluated regarding sensory properties and impurity content in order to establish its price. Only kernels severely contaminated with *Fusarium* spp. are classified into organic black impurity category, i.e. the category of deteriorated kernels. There is very little information on the distribution of the contaminated kernels during milling cleaning and the influence of milling processing on the safety of flour and other end-products. Particularly hazardous is preparation of wholegrain flour, because the whole kernels, including contaminated ones, are milled and processed into bread and bakery goods.

Each kernel category was analyzed using standard mycological, mycotoxicological, physico-chemical and rheological methods (Pitt and Hocking, 1985; Moresu, 1995; *Official Methods AOAC*, 1990; Kaluđerski and Filipović, 1998; *Pravilnik*, 1980; Marasas et al., 1984; Škrinjar et al., 1997).

RESULTS AND DISCUSSION

Target fungal species were detected in all of the investigated samples at varying levels according to the total fungal count per wheat kernel (Table 1). The highest contamination was observed in the category of grains severely contaminated with *Fusarium* spp. Overall, 9 fungal species were isolated, with *Fusarium* being the most dominant genus detected in higher numbers (78% of total isolated mycopopulations). There were eleven species (identified from *Fusarium* genus which could produce toxic metabolites like zearalenon (ZEA) and trihoteceron (Moresu, 1995; Marasas et al., 1984). *Fusarium oxysporum* was the most dominating mould, comprising 38% of the contaminated samples.

Table 1 — Average content of mold number per kernel of wheat fraction pattern

Grain fraction	1	2	3	4	5	6
Sound	0,92	0,73	0,83	1,15	0,75	1,11
Black germ	2,00	2,97	2,94	1,52	2,51	1,95
Moderatcly infested with *Fusarium spp.*	2,87	3,12	3.21	2,21	2,62	2,94
Severely infested with *Fusarium spp.*	3,21	3,25	3,25	2,58	3,21	3,14

Besides the most commonly isolated *Fusarium* spp., moulds from genus *Alternaria* were also present in the fraction of all the investigated wheat varieties, accounting for 14% of the samples (*Pravilnik*, 1980; Marasas et al., 1984; Šarić et al., 1997).

Investigating the physical properties of wheat fractions, it was concluded that test weights decreased depending on the infestation level (Šarić and Sekulić, 1981; Šarić et al., 2001) (Table 2, Figures 3, 4). Only fraction of sound kernels satisfied the minimum quality requirements regulated by JUS E.B1.200 standard (1992).

Table 2 — Physical properties of kernels from various fractions

Grain fraction	Test weight (kg/m^3)	Mass of 1000 kernels (g)
Average sample	710	27,4
Sound kernels	760	32,4
Black germ kernels	720	31,0
Moderatcly infested with *Fusarium spp.*	690	26,9
Severely infested with *Fusarium spp.*	550	19,4

Considering mycotoxicological analyses (Table 3), ochratoxin A (OA) was present in significant numbers of samples, mostly in the fractions of moderately and severely infested kernels with *Fusarium* spp., at concentrations in the range 11—48 mg/kg, while ZEA was detected in very high concentrations, ranging from 170 to 500 mg/kg. According to Regulation (*Pravilnik*, 1992), the maximum level of OA is 10 mg/kg, and 1 mg/kg for ZEA (*Official Methods AOAC*, 1990). These samples would be rendered unacceptable with respect to hygiene and safety, especially because toxins do not deteriorate after thermal processing (baking).

Tabele 3 — Contamination of wheat grains with mycotoxins (μg/kg)

Grain fraction	Variety	Mycotoxin (μg · kg^{-1})	
		Ohratoxin A	Zearalenon
Sound	1	0	0
Black germ	1	0	0
Moderatcly infested with *Fusarium spp.*	1	0	0
Severely infested with *Fusarium spp.*	1	8	280
Sound	2	16	0
Black germ	2	32	0
Moderatcly infested with *Fusarium spp.*	2	32	0
Severely infested with *Fusarium spp.*	2	32	0
Sound	3	32	0
Black germ	3	0	0
Moderatcly infested with *Fusarium spp.*	3	0	0
Severely infested with *Fusarium spp.*	3	32	400
Sound	4	0	0
Black germ	4	34	200
Moderatcly infested with *Fusarium spp.*	4	11,5	0
Severely infested with *Fusarium spp.*	4	34	500

Sound	5	11	0
Black germ	5	11	170
Moderatcly infested with *Fusarium spp.*	5	16	250
Severely infested with *Fusarium spp.*	5	48	350

Depending on the stage of kernel maturity, fungal infestations affect the filling of kernels, since the mass of 1000 kernels decreases regularly from sound to infested fractions. The kernel dimension is an important factor that affects flour yielding.

The highest flour yield was obtained in sound fractions, and the lowest in the severely infected fractions (Figure 3), that is in an inverse proportion with mineral content of kernels (Figure 4). The severely infested fractions showed the lowest flour yield, and the highest ash content, which is an unfavourable characteristic for milling processing.

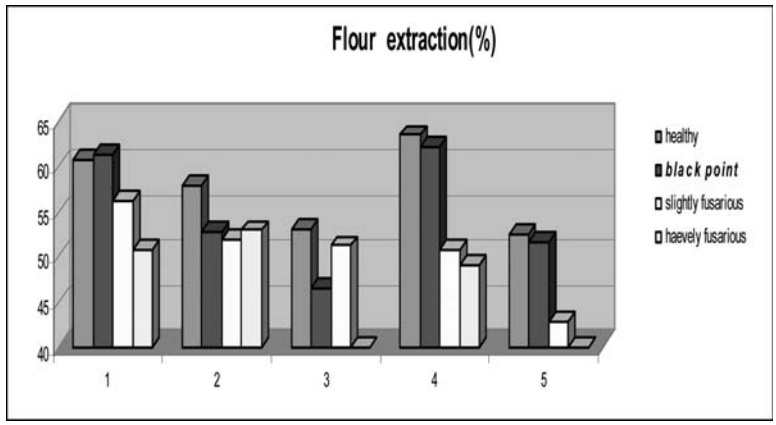


Fig. 3 — Flour yields of wheat fractions

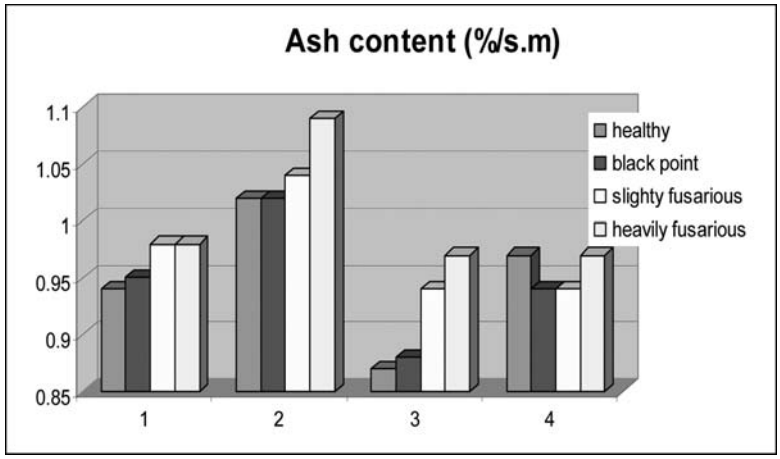


Fig. 4 — Mineral contents of wheat fractions

Chemical methods (protein content, wet gluten content, etc.) clearly revealed the breakdown of wheat gluten/starch conglomerate provoked by fungal infestation.

Table 4 — Protein content of wheat fractions

Variety	Protein content (%/dry basis)			
	Sound kernels	Black germ kernels	Moderatcly infested with *Fusarium spp.*	Severely infested with *Fusarium spp.*
1	13,3	13,7	13,8	14,0
2	13,6	14,0	14,5	15,0
3	14,0	14,6	14,8	15,1
4	14,8	15,0	14,9	15,5
5	14,3	14,8	14,9	15,1
6	13,5	14,0	14,3	14,8

All the investigated wheat varieties had relatively high protein content at a level of the first technological class, with the highest contents determined within the severely infested fractions (Table 4). This is the consequence of infestation with fungi belonging to genus *Fusarium* that resulted in prematuration and insufficient kernel filling, thus disturbing the ratio of endosperm to aleurone cell layer, in advantage to aleurone cell layer. This disturbance arose because moulds use carbohydrate components as a substrate for growth. Since the fungal biomass consists of 40% protein, the higher content of proteins in the infested fractions presumably comes from fungal mycelia present in significant amounts in the infested kernels.

From rheological measurements, the determination of quality and content of gluten was carried out. The wet gluten content significantly varied in the fractions of wheat varieties. The black germ fractions were the highest in this parameter, while the severely infested fractions were the lowest (Table 5). This is probably the consequence of gluten degradation, especially of gliadin, the major contributor to dough elasticity (Šarić et al., 1997).

Table 5 — Wet gluten content in wheat fractions

Variety	Wet gluten content (%)			
	Sound kernels	Black germ kernels	Moderatcly infested with *Fusarium spp.*	Severely infested with *Fusarium spp.*
1	32	33	30	26
2	33	35	31	27
3	34	36	32	30
4	35	37	34	32
5	35	36	33	29
6	34	33	31	26

Besides the difference in wet gluten content between fractions, there was a significant variation in the quality of gluten as well. Structural Berliner's number (Q_o) more or less regularly decreased from sound to the severely infested fractions of the examined wheat varieties (Figure 5).

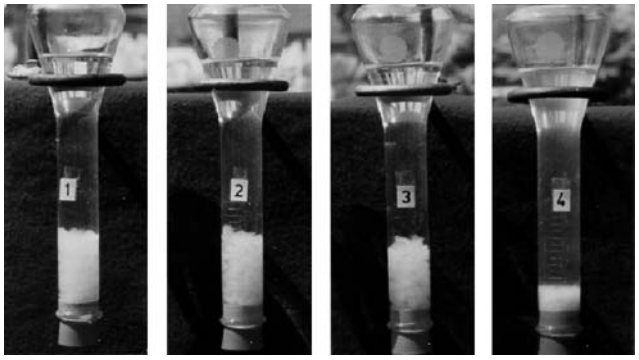
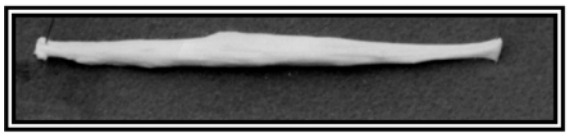


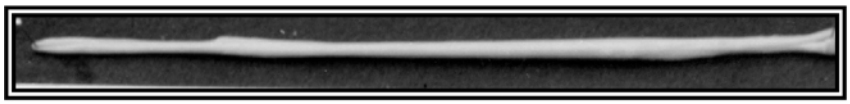
Fig. 5 — Changes in the structural Berliner's number (Q_0)

To investigate the gluten quality, gluten extensibility test was performed (Kaluđerski and Filipović, 1998). Gluten extensibility varied from fraction to fraction (Figure 6).

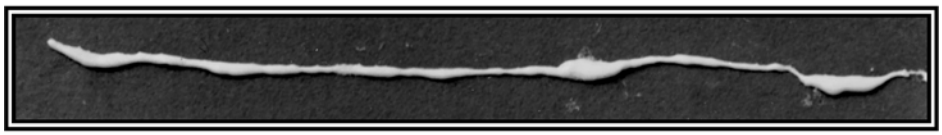
Sound grain fraction



Black germ grain fraction



Moderatcly infested with *Fusarium spp.* grain fraction



Severely infested with *Fusarium spp.* grain fraction

Fig. 6 — Changes in the properties of gluten

The sound fractions of all the investigated wheat varieties had moderately extensible gluten which was firm, slightly sticky, and elastic. The slightly infested fractions had moderately extensible, elastic, soft and mucous gluten. The

severely infested fractions had moderately to extremely extensible, non-elastic, sticky, and mucous gluten. In most of the cases, the incoherent mechanical structure enabled the gluten to return into the primary position after the extension.

CONCLUSION

— Moulds are frequent contaminants of wheat and related products with potential to produce mycotoxins;

— Fungal contamination by field moulds, especially from genus *Alternaria*, deteriorates the quality of wheat grains, depending on the level of infestation and the content of infested kernels;

— Field moulds, by their filaments, degrade the kernels, decreasing their processing quality and sanitary condition;

— Moulds and their metabolites, and mycotoxins cannot be detected organoleptically in grains, flour and bread, but they tend to accumulate in human organism, posing a health risk by causing severe diseases.

Especially emphasized is the contamination with moulds and their toxic metabolites which are resistant to heat degradation, thus representing potential silent killers of a living world. The basic recommendations coming from this paper are directed towards the strict enforcement of laws regarding microbiological and mycotoxicological control of cereals, as well as to encourage the changes and the necessary accommodation of valid regulations concerning toxin limits and introduction of obligatory control of newly recognized toxins.

ACKNOWLEDGMENT

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УТИЦАЈ ПЛЕСНИ НА ЗДРАВСТВЕНО БЕЗБЕДНУ ИСПРАВНОСТ И ТЕХНОЛОШКИ КВАЛИТЕТ *TRITICUM AESTIVUM*

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Резиме

Пшеница и прерађевине од ње намењене хуманој исхрани веома често подложне су инфекцији различитих плесни. Многи од њих су потенцијални продуценти широке лепезе микотоксина. Као последица напада плесни из родова *Fusarium* и *Alternaria* већином су губици у приносу, пад биолошког и технолошког квалитета, неподобност инфицираних зрна за производњу и прераду у људску храну због евентуалног садржаја микотоксина. Непознато је како и да ли се контаминирана зрна плеснима одвајају у млинској чистионици и распоређују у одређене типове брашна, као и у финалне производе. Интегрално брашно и производи од њега садрже све анатомске делове зрна, самим тим и микотоксине. Познато је да су микотоксини изразито термостабилни и не губе токсичност при термичкој обради, односно производњи финалних производа. Плесни из родова *Fusarium* spp и *Alternaria* spp синтетишу у зрну жита микотоксине и то већином зераленон и охратоксин А.

Циљ испитивања је био да се утврди присуство плесни у пшеничним зрнима. Посебно је важно да се изврши идентификација изолованих врста и то пре свега токсигених врста као и да се одреди њихов утицај на технолошки квалитет и здравствено безбедну исправност пшенице.

Испитано је шест сорти пшенице контаминираних плеснима и сви узорци су сензорно раздвојени на четири фракције зрна: здрава, тамноклична, мало и јако фузариозна.

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SHARE OF AFLATOXIGENIC MOULDS FROM GENERA *ASPERGILLUS* AND *PENICILLIUM* IN MYCOPOPULATIONS ISOLATED FROM SPICES FOR MEAT PROCESSING INDUSTRY*

ABSTRACT: Results of this paper show share of aflatoxigenic moulds from genera *Aspergillus* and *Penicillium* in mycopopulations isolated from spices that are often used in meat processing (ground pepper, dried peppercorn, paprika powder, caraway and laurel). Using standard mycological methods, it has been found that all the examined samples were contaminated by *Aspergillus* species, and further 50% by *Penicillium* species. Additionally, aflatoxigenic moulds *A. flavus* and *A. niger* were found to be present. *A. flavus* was present with 60% in the samples of dried peppercorn — 2; paprika powder — 2, while its frequencies in ground pepper — 1 and laurel were 50% and 28,5%. respectively. *A. niger* made up 50% of all the isolates of *Aspergillus* and *Penicillium* species, isolated from paprika powder — 1, although its share in the samples of laurel, paprika powder — 2 and ground pepper — 1 was 43%, 20% and 10% respectively.

KEY WORDS: aflatoxigenic moulds, frequency, spices

INTRODUCTION

Significant number of plants has been used for a production of spices because of its characteristical aromatic attributes. Basic role of these products is to make meals more tasty and piquant. Different parts of a plant could be used as a spice, depending on the part of the plant where aromatic substance is found. Such parts could be: offspring, seed, flower, leaf, cortex, root, or the whole plant (Dimić, 1999).

Foodstuffs of plant origin, to which species belong, are favourable substrate for development of mould. Besides the changes which are caused by

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some mould genera, like discoloration, in some definite conditions they can produce toxic extracellular metabolites-mycotoxins (K a r a n, 2005).

Production of spices is widespread in countries with tropical climate (high temperature and humidity, planty of rainfall), which is convenient for contamination with moulds. Besides, spices are usually dried on the ground in the open spaces with poor hygienic conditions. This further stimulates mould growth and production of mycotoxines. At the global level, contamination of spices with mycotoxines is most often registered in Ethiopia, Egypt and Portugal (Z i n e d i n e, 2006).

Spices are often contaminated with soil-borne moulds, and refuse (birds, insects, rodents). Number of moulds in spices increases because of inadequate drying process, while it reduces when water activity level is under 0.60. Xerophile moulds represent particular problem: they can develop at or in a substrate with smaller content of free water (under a_w 0,80) and mostly belong to genera *Aspergillus* and *Penicillium*, that are most frequent contaminants of spices (K a r a n, 2005)

Aflatoxins are products of a secondary metabolism of moulds *A. flavus* and *A. parasiticus*. Almost all species of *A. parasiticus* are toxic, but the synthesis of aflatoxins in *A. flavus* significantly varies between the species.

For both species production of toxins is a result of interaction of species genotype and medium conditions in which they grow (M o r e a u and M o s s, 1979). Besides the opinion that aflatoxins are secondary metabolites only of the mentioned mould species, there are opinions that other species also (*A. niger*, *A. ruber*, *P. citrinum*, *P. digitatum*, *Rhizopus stolonifer* and *Mucor mucedo*) have the ability for the aflatoxin synthesis, but only in traces (G o l d b l a t t, 1969; K r a s i ć, 2003).

Aflatoxins are secondary metabolites of moulds *A. flavus*, *A. parasiticus* and *A. nominus*, which is phenotype similar to *A. flavus*, but with different shape of sclerotium. The other species produce aflatoxins in traces, including *A. pseudotamarii*, *A. bonbysis* and *A. ochraceroseus* (E r d o g a n, 2004).

Aflatoxins are chemically difuro-cumaro-lactons. Until today, over 20 aflatoxins were isolated. Among them, the most famous and most toxic are the aflatoxins B1 and G1 and their dihydro derivates B2 and G2 (M a š i ć, 2000).

Aflatoxins are substances without colour, smell or taste. Chemically, they are stable and resistant to degradation during normal food cooking process. It is hard to eliminate them. They could be completely destroyed by chromsulfat acid, Na hipochlorite, concentrate NaOH and by being exposed to solar light for an extended period of time (Š u t i ć and S t o j a n o v i ć, 1973). They are dissolved without melting at temperature, from 268 to 269°C. Risk of producing aflatoxins is higher during dry season. When the humidity is under normal level and temperature is high, number of the spores of *Aspergillus* increases in the air (M a l e t i ć, 2005).

Aflatoxins are hepatotoxins and the most famous carcinogenic agents known until today (M a l e t i ć, 2005).

Toxic effect of aflatoxins, besides carcinogenic activity, includes mutagenic, teratogenic and immunosuppressive activities. AB1 is the most powerfull hepatocancer agent known in mammals and is classified by the International

Agency of Research on Cancer as Group 1 carcinogen (IARC, 1993). The incidence of AFs in foods and feeds is relatively high in tropical and subtropical regions where climatic conditions provide optimal conditions for the mould growth (R u s t o m, 1997). Furthermore, a correlation between dietary exposure to AFs and incidence of human liver cancer in some areas, especially in Africa and Asia has been shown (Z i n e d i n e i sar., 2006).

People are usually exposed to mycotoxins during food-consumption. Aflatoxins have cumulative activity in human organism and they cannot be eliminated by any known medicine.

The production of AB1 and AB2 has been registered at temperatures of 15, 20, 25, 30, 35 and 40°C by A d e b a j o et al. (1994), with optimal temperature of 30°C. At low temperatures (5°C), they are usually not produced (B u l l e r m a n and O l v i g n i, 1974; K r a s i ć, 2003).

Presence of aflatoxins is relatively rare in our country (M a š i ć, 2000). But, we are importing many foodstuff, including spices, from regions with tropical and subtropical climate that are often contaminated by aflatoxins. Therefore, it is clear that all the examinations of aflatoxins and their producers have great significance, especially if we know their nature and the fact that they are widely used in the food industry, particularly in the meat processing industry.

The main goal of this paper was to identify the share of aflatoxigenic moulds from genera *Aspergillus* and *Penicillium* in mycopopulations isolated from some spices.

MATERIAL AND METHODS

Mycological investigations were conducted on eight different spices samples intended for usage in meat processing industry, respectively on two ground pepper samples, two dried peppercorn samples, two samples of paprika powder and samples of caraway and laurel.

Total number of moulds in 1 g of a spice sample was determined according to Koch method. Three types of selective culture media were used: Sabouraud-maltose agar (SMA) with the addition of antibiotics (1,0 ml chloramphenicol and 1,0 ml oksytetracykline per 100 ml of medium), maltose yeast extract agar with 50% glucose (MY50-G), and medium for xerophile moulds-Czapek agar (Cz). The culture, dispersed in Petri dishes, was left to incubate for seven days at 25°C. Each test was repeated two times. The species isolated were monocultivated on Czapek agar, because of taxonomic classification. The samples were incubated for seven days at 25°C. The species isolated were identified on the basis of investigation of the macromorphological properties of colonies and micromorfological properties of conidial and other structures according to the key described by S a m s o n and van R e e n e n - - H o e k s t r a (1988), and T h o m and R a p e r (1945).

RESULTS AND DISCUSSION

Tab. 1 — Total number of moulds in 1 g of spice samples

Sample	Total number of moulds/1 g		
	CZ	SMA	MY50-G
Paprika powder — 1	200	320	680
Paprika powder — 2	680	820	1350
Ground pepper — 1	410	800	2520
Ground pepper — 2	120	530	1630
Dried peppercorn — 1	270	4040	6570
Dried peppercorn — 2	140	4210	1020
Caraway	10	0	3270
Laurel	780	810	2660

Tab. 1. shows results of total number of isolated moulds from spice samples.

The highest number of colonies in all spice samples, except dried peppercorn — 2 (4040 moulds/1 g of the sample, grown on SMA), was determined on MY50-G, and after that on SMA. The lowest number of colonies was determined on Cz, which was expected, because this is a medium that has the lowest content of nutrient compounds necessary for isolation of xerophil moulds. The presence of moulds on SMA was not determined in the caraway sample, after 7 days of incubation at 25°C.

The highest number of colonies on SMA was determined in the samples of dried peppercorn (4210 and 4040 moulds/1 g of the sample). The highest number of grown colonies on MY50-G was determined in the dried peppercorn — 1 sample (6570 moulds/1 g of the sample), and the most intensive growth of moulds on Czapek agar was observed in the laurel sample (780 moulds/1 g of the sample).

Tab. 2 — Species of moulds from genera *Aspergillus* and *Penicillium* isolated from spice samples

Sample	The species of moulds	Frequency of species (%)
Ground pepper — 1	*A. flavus* Link	50
	A. nidulans (Eidam) Wint.	10
	A. niger von Tieghem	10
	A. terreus Thom	10
	A. versicolor (Vuill.) Tiraboschi	10
	P. claviforme Bain.	10
Ground pepper — 2	*A. versicolor* (Vuill.) Tiraboschi	66,66
	P. corylophilum Dierckx	33,34
Dried pepper — 1	*A. ochraceus* Wilhelm	75
	A. versicolor (Vuill.) Tiraboschi	25
Dried pepper — 2	*A. flavus* Link	60
	A. ochraceus Wilhelm	20
	A. versicolor (Vuill.) Tiraboschi	20
Paprika powder — 1	*A. niger* von Tieghem	50
	A. ochraceus Wilhelm	50

Paprika powder — 2	*A. flavus* Link	20
	A. niger von Tieghem	60
	P. expansum Link	20
Laurel	*A. flavus* Link	43
	A. niger von Tieghem	28,50
	A. ustus (Bain.) Thom and Church	14,25
	P. glabrum (Wehmer) Westling	14,25
Caraway	*A. versicolor* (Vuill.) Tiraboschi	100

Tab. 2. displays the mould species from genera *Aspergillus* and *Penicillium* isolated from the investigated spice samples and their share in total number of mould isolates from the observed genera.

The presence of *Aspergillus* species was determined in all investigated spice samples. The presence of moulds from genus *Penicillium* was determined in the following samples: ground pepper — 1, ground pepper — 2, paprika powder — 2 and laurel.

Among aflatoxigenic moulds, the presence of *A. flavus* and *A. niger* was detected in the analyzed samples except the samples of caraway, dried peppercorn — 1 and ground pepper — 2. In the paprika powder — 1 sample, the only isolated aflatoxigenic mould was *A. niger*. Among the aflatoxigenic moulds only *A. Flavus* was isolated from the grain peppercorn — 2.

Fig. 1. shows the share of *A. flavus* in total number of isolates from genera *Aspergillus* and *Penicillium*, in the contaminated samples.

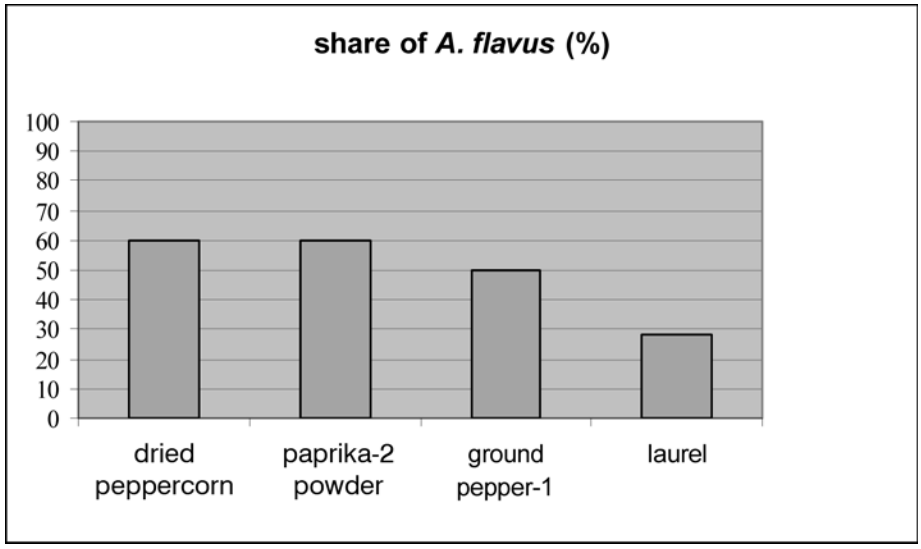

Fig. 1 — Frequency of *A. flavus* in mycopopulation of genera *Aspergillus* and *Penicillium* isolated from spice samples.

Fig. 2. shows the share of *A. niger* in total number of moulds from genera *Aspergillus* and *Penicillium*, in the contaminated samples.

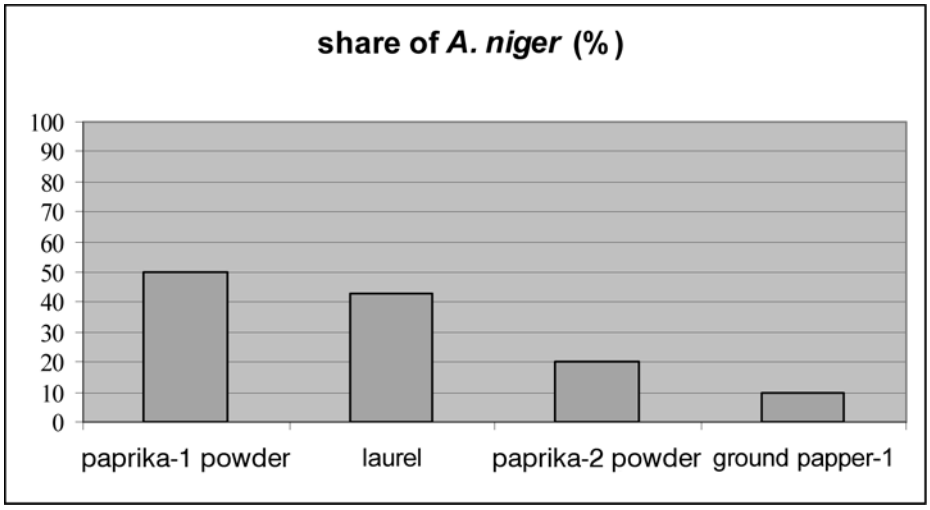

Fig. 2 — Frequency of *A. niger* in mycopopulation from genera *Aspergillus* and *Penicillium* isolated from the investigated spice samples.

Investigations performed by Hadlok (1969), Horie (1971), Sirnik and Gorišek (1983), Škrinjar (1983) and others, showed that the most frequent contaminants of spices were *Aspergillus* and *Penicillium* species (Dimić, 1999).

Dimić (1999) reported that 87,5% of the investigated spice samples (spice mixes, black peppercorn and paprika powder) were contaminated with moulds which belong to genera *Aspergillus* and *Penicillium.*

A. flavus detected in 50% of the mixed spice samples, and *A. niger* was determined in 62,5% of the same samples.

The results of mycological investigations on black peppercorn (Dimić, 1999) showed that the most numerous species of moulds were from genera *Aspergillus* and *Penicillium*, and they included 35,29% of all isolates. *Aspergillus* was detected in all analyzed samples (100%), and 62,5% of *A. flavus--oryzae* samples were contaminated by *Penicillium* species. The largest part of the investigated samples (75%) contained moulds which belong to group *A. flavus-oryzae*.

A. niger was isolated in 50% of black peppercorn samples. In paprika powder, *A. flavus* was shared with 25%, like *A. niger*, in total number of isolates (Dimić, 1999).

After investigation of 44 samples of paprika powder, Erdogan (2004) reported that 38,5% of the samples were contaminated with *A. flavus*. He detected the presence of *A. niger* in 57,7% of the examined samples.

Karan et al. (2005) detected the presence of *Aspergillus* in all the examinated spice samples, including dried peppercorn and paprika powder.

Misra (1981) and Roy et al. (1988) isolated high number of *Aspergillus* moulds from spices including black pepper (Karan et al., 2005).

CONCLUSION

It was found that all the examined samples were contaminated by *Aspergillus* species, and further 50% by *Penicillium* species. Additionally, aflatoxigenic moulds, *A. flavus* and *A. niger*, were found to be present. *A. flavus* was present in 60% of samples of dried peppercorn — 2; paprika powder — 2, while its frequencies in ground pepper — 1 and laurel were 50% and 28,5% respectively *A. niger* made up 50% of all the isolates from genera *Aspergillus* and *Penicillium* species isolated from paprika powder (regular), although its share in the samples of laurel, paprika powder — 2, and ground pepper — 1 was 43%, 20% and 10%, respectively.

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УДЕО АФЛАТОКСИГЕНИХ ПЛЕСНИ ИЗ РОДОВА *ASPERGILLUS* И *PENICILLIUM* У МИКОПОПУЛАЦИЈАМА ИЗОЛОВАНИМ ИЗ ЗАЧИНА ЗА КЛАНИЧНУ ИНДУСТРИЈУ

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Резиме

Резултати овог рада приказују удео афлатоксигених плесни из родова *Aspergillus* и *Penicillium* у микопопулацијама изолованим из зачина, који се често користе у индустрији прераде меса (млевени бибер, бибер у зрну, млевена зачинска паприка, ким и ловор). Користећи стандардне миколошке методе утврђено је да су сви испитивани узорци (100%) били контаминирани *Aspergillus* врстама, а 50% узорака и *Penicillium* врстама. Од афлатоксигених плесни утврђено је присуство *A. flavus* и *A. niger*. *A. flavus* је са 60% био заступљен у узорцима бибера у зрну — 2, млевене зачинске паприке (љуте), док му је учесталост у млевеном биберу — 1 и ловору била 50% односно 28,5%. *A. niger* је чинио 50% свих изолата *Aspergillus* и *Penicillium* врста изолованих из млевене зачинске паприке (слатке), док је удео у узорцима ловора, млевене зачинске паприке (љуте) и млевеног бибера — 1 био 43, 20 и 10%.

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FUNGAL DISEASES OF SOME VEGETABLES GROWN IN GREENHOUSE AND GARDEN*

ABSTRACT: Aim of this paper was to give an overview of fungal diseases prevailing on the most common vegetables grown in Novi Sad area. For investigation, lettuce and spinach grown in a greenhouse and in open garden were chosen. In greenhouse, optimal conditions for growing lettuce and spinach were maintained, which at the same time favour the development of fungal diseases. The vegetables grown in a protected suburban open garden were more problematic considering fungal diseases. In this paper, the prevention of fungal diseases was emphasized to avoid drastic chemical treatment or minimize its application. The adequate prevention in greenhouse is a good ecological measure.

KEY WORDS: Lettuce, spinach, greenhouse, fungal disease, fungus

INTRODUCTION

Lettuce and spinach grown in the greenhouse are different from field lettuce and spinach. Spinach contains other nutrients than just iron. Actually, the amount of iron in spinach comes way down the list after vitamins A and C, thiamin, potassium and folic acid (one of the B complex vitamins). Dark green leafy vegetables, like spinach, also contain lutein and zeaxanthin, both carotenoids. Studies have shown that carotenoids help our eyes stay healthy as we age by preventing macular degeneration and the formation of cataracts. Vitamins A and C, both antioxidants, keep our cardiovascular system healthy, thereby reducing the risk of strokes and heart attacks. Folic acid is essential for the production of red blood cells and for normal growth, and may reduce

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the risk of certain cancer types. It is particularly important for pregnant women.

Spinach, like lettuce, is a cool-weather crop; heat and long days cause the plants to bolt, which means that they produce flowering stems and seeds, and plants that bolt lose their flavour. These two crops require more exacting environmental conditions for optimum growth and quality than other vegetables. Seeds and transplants for greenhouse vegetables are expensive. These vegetables are usually started from seeds in small blocks of rock wool or similar inert substances. Transplants are then placed in the greenhouse to be grown to maturity. High quality transplants are critical for the success of greenhouse vegetable crops. It is strongly advised that growers produce their own transplants to avoid pests or pathogen contamination during transplant production or transport. Any production system requires diligent sanitation between crops. This involves removing old plants and bleaching, fumigating, or steaming production system components and growing surfaces.

Crop production and maintenance require several man/hours per greenhouse every day. A grower must be able to balance the demands of the greenhouse work load with demands from other occupations. Vacation days are hard to find for a greenhouse vegetable operator. Management operations include formulating fertiliser mixtures, monitoring irrigation systems, checking greenhouse operations, such as heating and cooling, ventilation, spraying for pest control, harvesting, packing, transporting and accounting book work. All of these operations must be done precisely on time.

The greenhouse should allow maximum photosynthetic ally active radiation during the daylight hours to support optimal plant growth. The greenhouse climate is warm, humid and wind free, providing an ideal environment for the germination of spores on any part of vegetables. The continuous use of *Benomyl* (methyl 1-(butilcarbamoil) benimidazol-2) and *Metalaxyl* (methyl N-(2,6 dimethylphenyl)-N-(metoxyacetyl)-DL alaninate) pesticides (fungicides) in a monocrop system (lettuce and spinach) exerts intensive selection pressure for pesticide resistant races of the pathogens. The appearance of a resistant strain and its further establishment and survival increase the risk of reinforcing resistant pathogen population by the renewed use of effective fungicides. It means to avoid the monocrop system and the use of only one kind of fungicide.

THE PROBLEM OF SUBSTRATE

In greenhouse there is a need for:
1. the substrate in greenhouse and
2. the substrate for production of nursery plant.

1. The space for the substrate in greenhouse is prepared by disinfection of soil with chemicals, or thermally, with water vapour. The disinfection is succeeded with cultivation and fertilisation. The common combination of fertilizer with fungicides does not give positive results.

2. In a separate room in the greenhouse the nursery plants are grown. If infested plants are transplanted, the disinfection of soil is automatically anulled.

In this work, for the protection of the nursery plants, "*Previcur N*" and thermical treatment with water vapour were used. It is recommended to use low cost waste as a fuel due to economical reasons.

Maintaining the Conditions in Greenhouse for Vegetable Production

The appropriate microclimate in greenhouse is one of the most important conditions. It requires the use of contemporary polyethylene (PE) sheets to avoid condensation of water vapour from air on the plants.

In winter time, the sunlight for photosynthesis must be supplied, and also the absorption of the UV spectrum 300—340 nm wave length. The technology with PE sheets was studied in detail in Israel, and they produce sheets with different absorbance. The effects are good, but for our economic situation this is not acceptable, even though the high technology of these sheets improves the quality of vegetables in practice.

Fungal diseases

In this paper, 16 fungal diseases on lettuce (Tab. 1) and 12 fungal diseases on spinach (Tab. 2) are presented (D a v i s, 1997). The mentioned diseases are the most important and widespread in this region of Europe. The fungal diseases on lettuce and spinach are most prevalent in cool and acidic soils, when water condenses on leaves and poor cultivation practice is used. In greenhouse, the prevention and control of fungi is more successful than in outdoor fields.

Tab. 1 — Lettuce diseases

No.	Fungal diseases	Fungus
1.	Alternaria leaf spot	*Alternaria sonchi*
2.	Anthracnose	*Michrodochium ponattonianum* = *Marssinina panattonana*
3.	Bottom rot	*Rhizyoctomia solani* *Thanatephorus cucumeris* [telemorph]
4.	Cercospora leaf spot	*Cercospora longissima*
5.	Damping-off, Pythium	*Pythium* spp. *Pythium ultimum*
6.	Damping-off, Rhizoctonia	*Rhizoctonia solani*
7.	Downy mildew	*Bremia lactucae,* *Plasmopara lactucae-radicis*
8.	Drop (Sclerotinia rot)	*Sclerotinia sclerotiorum,* *Sclerotinia minor*

9.	Gray mold	*Botrytis cinerea,* *Botryotinia fuckeliana* [telemorph]
10.	Phymatotrichum root rot (cotton root rot)	*Phymatotrichopsis omnivora* = *Phymatotrichum omnivorum*
11.	Powdery mildew	*Erysiphe cichoracearum*
12.	Rust	*Puccinia dioicae* = *Puccinia* *extensicola* var. *hieraciata*
13.	Septoria leaf spot	*Septoria lactucae*
14.	Southern blight	*Sclerotium rolfsii* *Athelia rolfsii* [telemorph]
15.	Stemphylium leaf spot	*Stemphylium botryosum* *Pleospora tarda* [telemorph]
16.	Wilt and leaf blight	*Pythium tracheiphilum*

Tab. 2 — Spinach diseases

No	Fungal diseases	Fungus
1.	Anthracnose	*Colletotrichum dematium* = *Colletotrichum spinaciae*
2.	Aphanomyces root rot	*Aphanomyces cochlioides,* *Aphanomyces cladoganus*
3.	Cercospora leaf spot	*Cercospora beticola*
4.	Downy mildew = blue mold	*Peronospora effusa*
5.	Fusarium wilt	*Fusarium oxysporum* f. sp. *spinacia*
6.	Leaf spot	*Alternaria* spp. *Ascochyta spinacia,* *Cercospora* spp. *Cladosporium* spp. *Myrothecium* spp. *Phyllosticta* spp. *Ramularia spinacia*, Other fungi
7.	Phytophtora root rot	*Phytophtora cryptogea,* *Phytophtora megasperma*
8.	Pythium root rot	*Pythium aphanidermatum,* *Pythium heterothallicum,* *Pythium sylvaticum*
9.	Red rust	*Puccinia aristidae,* *Aecidium capsicum* [anamorph]
10.	Seed mold	*Alternaria* spp. *Curvularia* spp. Other fungi
11.	White rust	*Albugo occidentalis*
12.	White smut	*Entyloma ellisii*

Many fungi prevalent in soils can cause damping-off. *Fusarium* spp., *Pythium* spp., *Phytophtora* spp. are most active in cool, wet soils, whereas *Rhysoctonia* spp. are more active in warm, wet soils (R a i d, 1997).

Damping-off can be controlled with proper cultivation practices: seeds should be planted on well-drained sites or in raised beds at soil temperatures above 15°C, avoiding dense stands. Nitrogen fertilizers should not be applied until seedlings are 6 weeks old. Soil acidity should be maintained at pH 6.0, or slightly above. Soil fumigation or seed treatment with fungicides is sometimes necessary for adequate control.

Diseases of Lettuce

There are three major fungal diseases of lettuce grown in greenhouses: *sclerotinia drop* (S u b b a r a o, 1997), *bottom rot* and *downy mildew*. All are favoured by moist conditions, although bottom rot is favoured by warm and moist conditions, while others are favoured by cool and moist conditions (K e r n, 2006). All produce fungal growth under wet conditions that allow diseases to be distinguished (K o i k e, 1997).

Sclerotinia Drop

Sclerotinia drop is caused by the fungi *Sclerotinia sclerotiorum* and *S. minor*. These fungi affect lettuce and many other plants, including spinach. Sclerotinia drop is a serious disease. It is now believed to be found in greenhouses wherever there is cool and moist. Under moist conditions, the entire plant may collapse in a few days.

Characteristic symptoms: The first symptom is wilting of the outermost leaves. Before the leaves wilt, a water soaked area causes the fungus to grow, and it appears on the stem near the soil. As the fungus will grow from this point down into the roots and up through the rest of the stem, it will also grow into each leaf, causing the base of the leaf to rot. This causes the leaves to drop and wither, and their tips to touch the soil, or rest on leaves below. As the fungus grows up the plant, each leaf is affected in turn. The inner leaves usually remain moist enough for the fungus to invade them completely and reduce them to a slimy mass. Under moist conditions, a snowy white fungus mass will bi produced over the entire head. Black structures, as small as a mustard seed, or as large as a bean, may be formed in this web of fungal growth, usually on the undersides of the leaves touching the soil.

Identification of Sclerotinia drop: snowy white web-like fungal growth is present.

Bottom Rot

Bottom rot is caused by the fungus *Rhizoctonia solani*, which affects lettuce and many other fleshy plants. It is now a greenhouse and field disease and is favoured by warm, wet conditions. Plants are usually affected when they are nearly mature.

Characteristic symptoms: The first symptom seen from above is usually wilting of the outer leaves. The fungus enters the plant through lower leaves which are touching the soil. Spots appear on the leaf petioles and midribs, and they can be very small or can grow rapidly to cover the entire petiole/midrib area. Spots may ooze a light brownish or amber colour liquid. Under warm, wet conditions, the fungus will continue to grow upward into the leaf blades and destroy them, as it grows from leaf to leaf. The stem is usually the last part of the head to decay. The fungus also provides a path for the entry of secondary rot bacteria.

Identification of Bottom rot: tan to brown web-like fungal growth on plant.

Downy Mildew

Downy mildew is caused by *Bremia lactucae* Reg. which attacks lettuce and numerous other vegetables.

Characteristic symptoms: The affected leaves lose their natural green colour and turn yellow. A careful examination will disclose a downy web on the lower side of the foliage which will have a wilted appearance. The downy web consists of the conidiophores of the fungus. These appear single and are much branched. The conidia germinate by means of a germ tube. Downy mildew is a disease which is troublesome in Europe. It is more serious on greenhouse lettuce than on that grown in the open. Downy mildew attacks not only lettuce, but also chicory and numerous other Composite.

Control: This disease is controlled by use of systematic fungicide *Benomyl*.

Fungal Diseases of Spinach

Downy mildew, a fungal disease, is the primary disease of spinach (S u m m e r, 2006). It produces slightly yellow or chlorotic lesions of irregular shape on the top surface of the leaves, and purplish sporulation on the underside. In order to prevent it, plants should be set apart for good air circulation and, when watering, the plant's foliage should not get wet. To help avoid soil borne diseases, such as *Rhizoctonia*, *Pythium* or *Fusarium*, the plantings should be rotated each year; in other words, spinach should not be sowed in the same row or bed every year (d u T i o t, 2005).

White Rust

This fungal disease is caused by the fungus *Albugo occidentalis* and is the major disease of spinach. In advanced stages, the white lesions form on the upper side of the leaf.

Characteristic symptoms: Plants infected with white rust fungus are weak and collapse quickly if environmental conditions are favourable for disease development. In summer, the fungus in soil is dormant, and its thick-walled

oospores may spread within a field by windblown spores. Free moisture on the leaf surface must be present for spore germination and development. The optimum temperature for germination is 30°C. The disease develops most rapidly at 40°C, or during periods of cool, humid nights and mild day temperatures.

Identification of white rust: Only the upper surface will be chlorotic (Olsen, 1998).

Downy Mildew or *Blue Mold*

Downy mildew or blue mold is caused by fungi: *Peronospora effusa* and *Peronospora spinaciae*. The underneath side of the leaf is marked by grey to violet-grey fungal growth mat that bears sporangia. The entire leaf is killed by susceptible varieties under optimum environmental conditions. In winter, the fungus remains in living spinach plants and in the seed. The fungus spores require surface moisture for development. Optimum temperature is around 27°C for germination and 30 to 35°C for development.

The disease mainly develops in mild, humid (dew) conditions, in young plants. Sporulation and germination of sporangia occur between 9 and 12°C. Seedlings are easily infected; adult plants are susceptible to infection while growing, if free water is present for at least 6 hours.

Symptoms: Spots of downy mildew on leaves of spinach.

Identification of Spinach downy mildew: Downy cover of downy mildew on the leaves of spinach.

Damping-off and Root Rot

These diseases are caused by pathogens: *Fusarium oxysporum, Pythium species* and *Rhizoctonia solani*. Damping-off is problematic in spinach production areas throughout the world. Severity is influenced by cultivar, soil texture, irrigation management and pathogen populations. Severe damping-off is associated with clay or poorly draining soils. Although spinach can be infected by root rot organisms in all its growth stages, newly emerging plants and young seedlings are especially susceptible.

Characteristic symptoms of damping-off and root rot: Stunted plants, yellow lower leaves, general poor growth, wilting and eventual collapse and death of plants. Roots of infected plants can appear water-soaked or brown to black in colour. The upper taproot may be girded by a necrotic lesion, or the tip of the taproot may be necrotic. In severe cases, nearly all roots may be girdled.

MATERIALS AND METHODS

In this work, lettuce (*Lactuca sativa*), type: Butterhead lettuce, variety "*Atrakcija*"; and spinach (*Spinacia oleracea*), type: Plain Leaf, variety "*Viroflay*" were investigated. These vegetables were grown in greenhouse, in experimental and control groups.

Lettuce was produced in PVC containers (black coloured) produced by TEKU. Thermally disinfected compost substratum was used. After 35 days, the seedlings were transplanted in a separate room. The seedlings were moistened with diluted nutrient solution until they were ready for transplantation. The nutrient was with low nitrogen content, KEMIRA, *Ferticare IV*.

Spinach was produced from seeds (ZKL, protected seed) 3500 plants (arranged on 5 x 15 cm) were fertilized with KEMIRA, *Ferticare IV*. The humidity of the substratum was maintained under optimal conditions, the temperature of water was 12°C. The experimental plants were also protected with "*Agril folija*" sheets (19 g/m^2) during cool nights. The "*Agril folija*" sheet protects up to –5°C.

The experimental groups of lettuce and spinach were treated with systematic fungicide *Benomyl* (Hinoin — FUNDAZOL® 50WP, 50% Benomil, Agrochem, Budapest). The identification of fungal diseases was done by means of practice and morphological characteristics.

Lettuce and spinach diseases were identified on the basis of visual observation of fungal diseases symptoms, and on the basis of comparison of our photos of fungal diseases (Figures 1—3) with some photos from the Slide Show (1—3):

Fig. 1 — Infected and sound spinach
(Photography: Michael Pajkert)

Fig. 2 — Lettuce infected with *Bremia lactucae* Reg
(Photography: Michael Pajkert)

Fig. 3 — Greenhouse with lettuce and spinach
(Photography: Michael Pajkert)

Slide Show 1: 2006 International Spinach Conference, 13—14 July 2006, La Conner, Skagit Co., WA; Author of Slide Show Lindsey du Toit, WSU (Washington State University), Mount Vernon NWRE, Organic Seed Alliance, published on the occasion of Spinach Scad Field Day 23 May 2006, Seguin WA (http://Caps.WSU.Edu/Conference/Spinach.

Slide Show 2: http://edis.ifas.UFL.Edu/VH044 from authors: Raid and Datnoff and

Slide Show 3: http://www.hydromall.com/grower/mildew_diseases.html from author: Dr. Lynette Morgan.

DISCUSSION

The fungal diseases were identified on lettuce in greenhouse experiment (Figure 3). Figure 2 shows the appearance of sclerotinia drop caused by *Sclerotinia sclerotiorum* and *Sclerotinia minor*. Rust rot by *Alternaria zinniae*, downy mildew, caused by *Bremia lactucae* and *Plasmopora lactucae-radicis* was also diagnosed. Downy mildew is a foliar disease. Cool and moist conditions are necessary for fungal development. Free moisture on the leaf surface is essential for spore germination and infection. Initial symptoms are yellow ribbon on upper side of a leaf on butterhead lettuce. There was also leaf spot disease and rust fungus on leaf, but in a very small number of lettuce, because of good every day control. None of the plants was infected with *Botrytis cinerea*, nor was grey mold identified.

Spinach was also grown in the greenhouse and treated with benomil (Figure 3). The humidity of soil was maintained without moistening the plants. Among 3500 plants, one sample was found to be infected with spinach mosaic virus, and as a secondary phenomenon appeared root rot, caused by *Pythium* spp. (Figure 1). Identification of these fungal diseases was done usually, by means of comparative study of digital photographs of plants and the Slide show 1 of spinach, affected by fungal diseases.

CONCLUSION

Lettuce and spinach investigated in this work were susceptible to fungal disease.

On the lettuce *Sclerotinia sclerotiorum* and *Sclerotinia minor*, which caused sclerotinia rot, were found. Also, *Alternaria zinniae* and *Septoria lactuca* leaf spots were found. *Bremia lactucae* and *Plasmopora lactucae radicis* were also identified, which caused downy mildew in experimental and control group, respectively.

Only one spinach plant, of 3500, was infected with viruses, and as a secondary appearance, rust rot caused by *Pythium* spp. in experimental group was observed.

Spinach could be produced without fungicides, scientific farming methods if were used.

The production of lettuce is not possible without a complete chemical plant protection.

The spinach is one of the most suitable vegetables from the ecological view point.

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ГЉИВИЧНА ОБОЉЕЊА НЕКОГ ПОВРЋА У СТАКЛЕНИЦИМА И У БАШТАМА

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Резиме

Циљ овог рада је да прикаже учесталост гљивичних обољења оног поврћа које се најчешће гаји у околини Новог Сада, у стакленицима и у баштама. За испитивање су одабрани салата и спанаћ. Стакленици обезбеђују оптималне услове за раст салате и спанаћа, а у исто време су ти услови погодни и за појаву гљивичних обољења. Заражене биљке треба уклонити. Код поврћа које се узгаја у отвореним баштама лакше се јављају гљивична обољења. У овом раду се истиче превенција гљивичних обољења, са циљем избегавања драстичних хемијских третмана, односно да се њихова употреба сведе на минимум. Под адекватном превенцијом, у стакленицима, остварују се добре еколошке мере за раст здравих биљака.

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FUSARIUM ROT OF ONION AND POSSIBLE USE OF BIOPRODUCT*

ABSTRACT: Several species of *Fusarium* are causal agents of onion rot in field and storage. Most prevalent are *F. oxysporum* f. sp. *cepae* and *F. solani*, and recently *F. proliferatum*, a toxigenic species. Most frequently isolated fungi in our field experiments were *F. solani* and *F. proliferatum* with different pathogenicity. Certain differences in antagonistic activity of *Trichoderma asperellum* on different isolates of *F. proliferatum* and *F. solani* have been found in *in vitro* study in dual culture, expressed as a slower inhibition of growth of the former, and faster of the latter pathogen. Antagonistic abilities of species from genus *Trichoderma* (*T. asperellum*) are important, and have already been exploited in formulated biocontrol products in organic and conventional production, in order to prevent soil borne pathogens inducing fusarium wilt and rot. The importance of preventing onion infection by *Fusarium* spp., possible mycotoxin producers, has been underlined.

KEY WORDS: *Allium cepa* (onion), antagonism, bioproduct, *Fusarium* rot, *Trichoderma asperellum*

INTRODUCTION

Several species of *Fusarium* are associated with rot and deterioration of onion in field and storage. Most often, *F. oxysporum* Schlecht. f. sp. *cepae* (H. N. Hans.) W. C. Snyder & H. N. Hansen causes the rot of a basal plate, *F. solani* (Mart.) Appel & Wollenw. in early growth stage, but recently, the attention has been paid to *F. proliferatum* (Matsushima) Nirenberg, as a toxigenic species producing important mycotoxins. Only few reports on *Allium* disease caused by *Fusarium* species, in section Liseola, were published. *F. proliferatum* was reported on onion in Italy (Manneruccі et al., 1987), and in Germany as mycotoxin producing fungus in garlic (Seefelder et al., 2002).

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As a Causal agent of garlic bulb rots, it is also capable of rotting actively growing plants, but also curing garlic bulbs, as it was reported in North America. The same year, du Toit and Inglis (2003) reported this pathogen on 70% of the harvested bulbs of white varieties, but not on yellow and red onion in Columbia Basin of Central Washington and Dugan et al. (2003) reported it as a pathogen of serious impact on garlic and onion in the Pacific North West. Because of documented mycotoxin production, increasing disease pressure and currently unknown etiology, the documentation of instances of *F. proliferatum* attacking bulbs is strongly warranted.

In Serbia, the presence of *F. proliferatum* on garlic and white onion varieties was observed in 2000 (Stanković et al., 2005), and fumonisin B_1, beauvericin and fusaproliferin producing strains were confirmed (Stanković et al., 2007).

Chemical measures in management of soil borne pathogens, especially those infecting onion in late vegetative phase, are of limited or no value. Therefore the presence of root colonizing antagonist seems acceptable for many reasons, including self propagation, longer persistence in soil environment, and ecological and toxicological benefit for workers and consumers.

Many *Trichoderma* strains have been identified as having potential applications in biological control, and eight of commercial products (Bio-Fungus, Belgium; Root Pro and Trichoderma 2000, Israel; Trochoject, Trichopel, Trichodowels and Trichoseal, New Zealand; TUSAL, Spain; Trieco, India; Trichodex, Hungary) are available against pathogenic *Fusarium* species (Monte, 2001).

The objectives of this study were: (i) determination of main *Fusarium* species causing onion rot; (ii) evaluation of their pathogenicity on onion under ambient laboratory conditions; (iii) study of potential antagonistic effects of *Trichoderma asperellum* Samuels, Lieckfeldt, Nirenberg (teleomorph *Hypocrea asperella* Starbäck) on *Fusarium* species.

MATERIAL AND METHODS

Isolation of pathogens

Onion bulbs were inspected for possible presence of *Fusarium* species. The samples from diseased tissue were disinfected in 0.1% sublimate solution, rinsed in sterile water and planted onto potato dextrose agar (PDA) amended with streptomycin sulphate (50 mg L^{-1}). The isolates were purified and underwent preparation for determination.

The fungi were single-spored and sub-cultured on PDA under permanent darkness at 25°C, on carnation leaf agar (CLA), and on nutrient synthetic agar (SNA) under an alternating temperature of 25°C during day and 20°C at night, with 12 h photoperiod. On the basis of morphological characteristics, *Fusarium* isolates were identified according to Gerlach and Nirenberg (1982), Nelson et al. (1983) and Burgess et al. (1994).

Pathogenicity test

Artificial infection of onion sets (disinfection, wounding, immersion in spore suspension) (16 sets/repetition) was done according to du Toit and Inglis (2003), incubated in moist chamber at 20°C and examined daily for symptoms during two weeks.

After two weeks, onion sets were rinsed in water, surface sterilized for 10 min in 0.5% sodium hypochlorite, and cross-sectioned to visualize punctures. After incubation 2-day mycelia was transferred to solid PDA medium amended with streptomycin sulphate.

The used isolates and re-isolates were inoculated on PDA in the same Petri dish and compared.

Antagonistic experiments in vitro

T. asperellum, active moiety of biological product Trifender (BioVed), ability to antagonize onion pathogens *F. solani* and *F. proliferatum* (obtained from field experiment) was tested in laboratory experiment under controlled conditions.

T. asperellum antagonism was investigated in dual culture plates against tested phytopathogenic *Fusarium* species. The experiments were conducted in Petri dishes on solid PDA in darkness at 25°C the temperature which enables growth of both pathogens and *T. asperellum.* Agar plates (10 ml per Petri dish) were inoculated with agar disks (5 mm in diameter), cut from the edges of *Fusarium* colonies growing on solid PDA medium. Two days after the first inoculation, *Trichoderma* was inoculated in the same way 6 cm apart from the position of the first inoculum. The plates were incubated at 25°C in darkness, for two to three weeks, and examined daily for growth rates and morphological characteristics, such as colony appearance, colony diameters, sporulation and pigment formation.

RESULTS AND DISCUSSION

Determination of Fusarium species

After undergoing the mycological procedure, *F. proliferatum* and *F. solani* were found to be the most frequently isolated species. Three isolates of each — *F. proliferatum* (Figures 1, 3, 4) and *F. solani* (Figures 2, 4, 6) were selected for pathogenicity test and study of antagonistic effect of *T. asperellum* (Figures 7, 8).

Pathogenicity of F. proliferatum and F. solani on onion bulbs

Re-isolation of the fungal strains confirmed that the symptoms on the infected sets were caused by corresponding isolates of *F. proliferatum* or *F. solani* used for inoculation.

In Tab. 1, the percent of sprouted onion sets, the percent of sets with long sprouts, and of *Fusarium* positive are given. The results from dual culture growth rate of fungi both confronted and non-confronted, as well as days spent to meet colony edges or to overlap, are given in Table 1 and in Figured 9—14.

Tab. 1 — Effects of *F. solani* and *F. proliferatum* on onion sets after artificial infection and antagonistic effect of *Trichoderma asperellum* in dual culture experiment

Fusarium spp.	Isolate No	Percentage of onion sets			overgrown by *T. asperellum* in	
		sprouted	long sprout	diseased	days	cm
F. solani	F6IIB	81,3	25	43.8	7—11	0.2—2.2
F. solani	FKIIIA	50	12,3	87.5	7—11	1.1—2.2
F. solani	F 5/IV bel	50	25	100	5—40	0.5—2.5
F. proliferatum	FKIII dan	18.8	6.3	100	7—40	0,5—1
F. proliferatum	F 8/I	37.5	18,8	81.3	6—8	0.7—1
F. proliferatum	F 3/IIIB	43.8	18.8	100	10	1.0
Noninfected		81.3	56.3	12.5		

Our research confirmed the pathogenicity of *F. proliferatum* on yellow cultivars opposite to du Toit and Inglis results (2000) who isolated pathogens from white cultivars. The differences were found between the isolates of both pathogens: in the percent of the diseased and sprouting sets (Tab. 1, Fig. 15—20). The highest number of well-developed sprouts appeared in non-infected control sets, that underwent the same procedure, but with sterile water immersion. Very aggressive isolates infected 100% of onion sets. When compared to the percentage of positive infected sets, *F. proliferatum* isolates with low overgrowth in dual culture test, appeared highly pathogenic and inhibited sprouting from 18.8 to 50.0% (Table 1). The high percent of set infection coincided with lowest percent of sprouting and sprout elongation, and in dual culture test there was minimum inhibition of *F. proliferatum* isolate FK III "dance", and no inhibition in case of isolate F 3IIIB by *T. asperellum*.

Fusarium species inhibit the sprouting and sprout elongation. *Trichoderma* species are known as being capable of deactivating some enzymes formed by pathogens. These traits should be taken into account when judging the influence on sets sprouting.

Antagonistic activity of Trichoderma asperellum in vitro

Results of comparing the antagonistic activity of *T. asperellum* and different isolates of *F. proliferatum* and *F. solani* indicated different degrees of antagonism. Mycelia growth was affected to different degrees, depending on specific interactive microbial couplet.

More days were needed for *Trichoderma* species to approach *F. proliferatum* than *F. solani* colonies. *F. solani* colonies were overgrown by myce-

lium of *T. asperellum* to a higher extent, in comparison to *F. proliferatum* isolates, of which some were not completely overgrown even after 5 weeks.

The investigated strain of *T. asperellum* was able to overgrow the isolates of *F. solani* mycelia and produce conidia on their surface, indicating mycoparasitic action (Figures 21—23).

In case of *F. proliferatum* isolates the difference in *T. asperellum* mycelia growth was found (Figures 24—26). The isolate F K-III dance of *F. proliferatum*, during the antagonistic interaction, produced distinct colour at the confronting colony edge (Figure 26). In some cases, a clear inhibition zone was visible and radial growth pathogen was limited to small extent (Figure 24), whereas for isolate F 8/I of *F. proliferatum*, two concentric rings of sporulation were formed in the zone of confrontation (Fig. 25).

T. asperellum decreased the rate of mycelia growth of *F. solani* and *F proliferatum*. However, its ability to completely overgrow the colony was demonstrated only with *F. solani* isolates. It took five days to grow to the edge of the colony, and eleven days to overgrow the same. However, after short period of growth retardation, *T. asperellum* grew over the colony of *F. proliferatum* to a certain limit, but never completely until after 40 days (Table 1).

One could speculate that *T. asperellum* is capable of mycoparasitism of *F. solani* but not of *F. proliferatum.* The consequence could be a less powerful protection against *F. proliferatum* infection. Speculations on other employed suppression mechanisms ought to be confirmed in future experiments, to explain the good effect of *T. asperellum* bioproduct on onion protection in field.

The experiments were conducted at 26°C in darkness, at temperature optimal for growth of both pathogens and *T. asperellum*, as it was known from the literature data that *T. asperellum* prefer swarm soil conditions. Its optimum growth *in vitro* is at 30°C. *Fusarium* species are also growing well on this temperature, and infection of bulbs occurs late in the season, often aided either by some mechanical or insect injury, or by water or heat stress in soil. Therefore we speculated that, once present in soil, *T. asperellum* would develop and overgrow the bulb roots, preventing infection of *Fusarium* species. Once it establishes in the bulb microenvironment, it would significantly protect root and bulb from pathogens.

The differences in behavior in dual culture between the isolates determined as *F. proliferatum* are of interest for clarifying their physiological profile. In future experiments, the isolates should be tested to detect the nature of specific reaction.

Not only one mechanism is involved in *Trichoderma — Fusarium*, or other pathogens interaction — from competition for root exudates to fungi super parasitism. The nature of this particular relation, in case of onion pathogen, remains to be further clarified.

T. asperellum has been recently shown to induce systemic resistance in plants through a mechanism that employs jasmonic acid and ethylene signal--transduction pathways. *Trichoderma* activates plant defence mechanisms, which results in infection suppression the leaf pathogen *Pseudomonas syringae* pv. *lacrymans* (Smith & Bryan) Young et al (S h o r e s h et al., 2006).

Suppressive effect of *T. viride* (Pers ex Gray) Gorenz strain B35 on onion pathogen *Pyrenochaeta terrestris* (Hansen) was explained by production of extra cellular hydrolytic enzymes (protease, cell wall degrading enzymes), (Pietr, et al., 2004). Applied as a bulb treatment before planting, it increased significantly the marketable yield, during three following seasons. The observed stimulating effect on the growth suggested the induction of systemic resistance, resulting in soil-borne pathogen suppression.

Managing the health of onion was the focus of many investigations, due to poor chemical protection from soil pathogens, such as *Fusarium* and *Sclerotium* species. Products accepted even in organic vegetable production are based on several biocontrol organisms, such as *Trichoderma* species and *Coniothirium minitans*. *T. harzianum*, Rifai T-22HC and T22-Planter Box (Bio Works), formulated for broadcast seed treatment, in-furrow spray and transplant starter. They extand root protection beyond chemical seed treatment: protects roots against diseases caused by *Pythium*, *Rhizoctonia* and *Fusarium* species and create stronger root systems.

Biocontrol agent use is justified by its persistence and self propagation in soil, different modes of action to pathogen, activation of resistance, low toxicity for mammals, consumers and field workers, beneficial effect on root and plant development, permission to use in organic production, etc. Disadvantages are short shelf life, in most cases sensitivity to environment effects, and necessity for know how education of farmers.

Fusarium rot are hard to control being seed and soil-borne, long persistant (over 4 years), capable of infection and spread in field and storage. So far, the best solution is the use of resistant short-day and intermediate onion varieties. Therefore integrated measures: cultural, breeding and biological or chemical, are the only justified approach to onion protection nowadays.

Fusarium species infecting onion, affect the health safety of agricultural workers, especially those associated with processing and store houses, as well as the consumers. Fumonisin B_1, beauvericin and fusaproliferin producing strains were confirmed as products of *F. proliferatum* on garlic and white onion variety in Serbia (Stanković et al., 2005, 2007). However, the other *Fusarium* species are of interest as well. Onychomycosis is usually caused by *F. solani* and *F. oxysporum*, but Hattori et al. (2005) reported *F. proliferatum* as a causal agent of onychomycosis for the first time. It was reported to cause suppurative thrombophlebitis in an immunocompromised patient (Murray et al., 2003), endophalmitis after cataract surgery (Ferrer et al., 2005), and disseminated infection in a child with lymphoblastic leukemia (Summerbell et al., 1988). Use of bioproducts would greatly contribute to an improvement of health safety of both producers and consumers.

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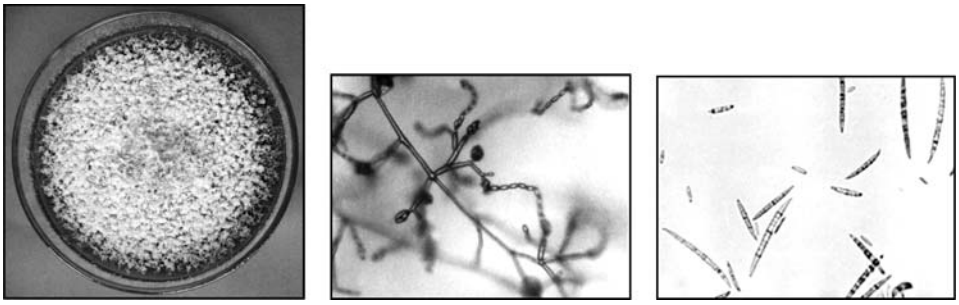

Fig. 1, 3, 4 — Colony of *F. proliferatum* on PDA and morphological characteristics

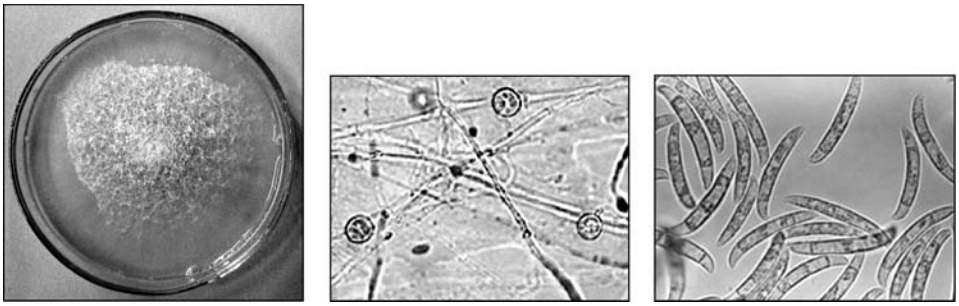

Fig. 2, 5, 6 — Colony of *F. solani* on PDA and morphological characteristics

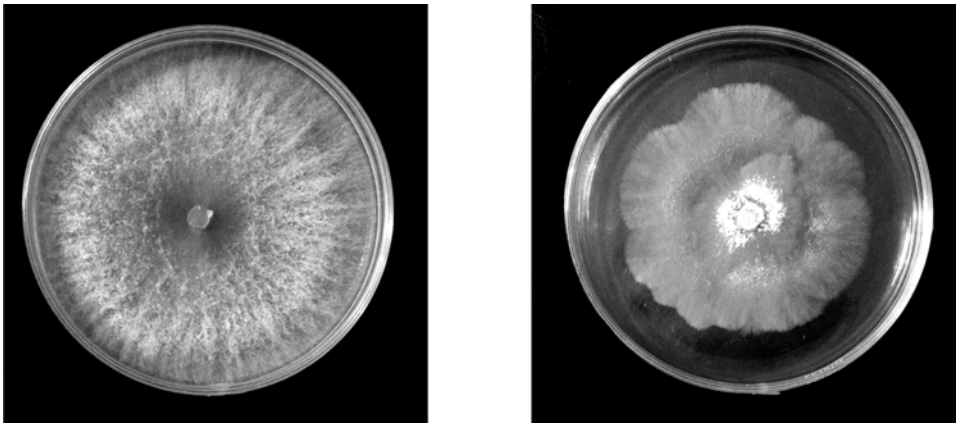

Fig. 7. and 8 — *Trichoderma* colony on PDA not amended (7) and amended (8) with Streptomycin sulphate at 50 ppm

Fig. 9. *Fusarium solani* isolate F 6-IIB

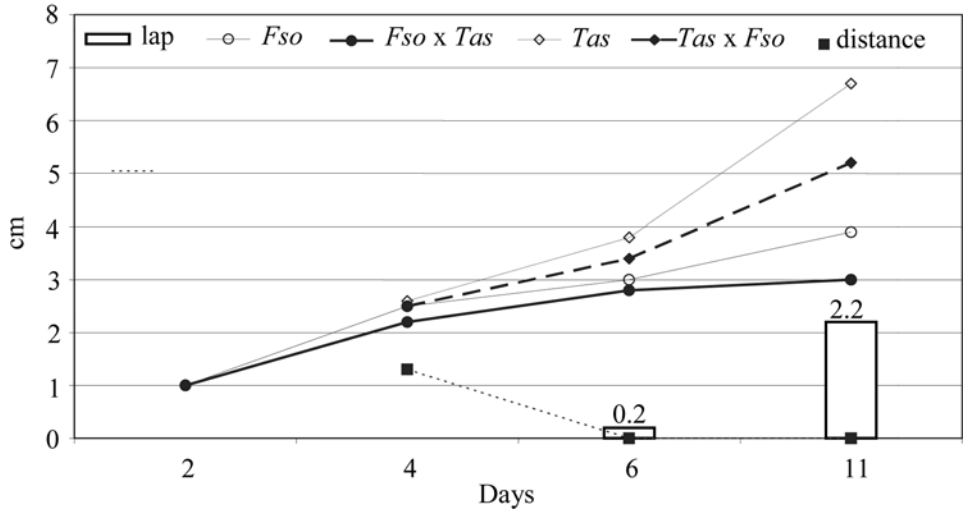

Fig. 10. *Fusarium solani* isolate FKIIIA

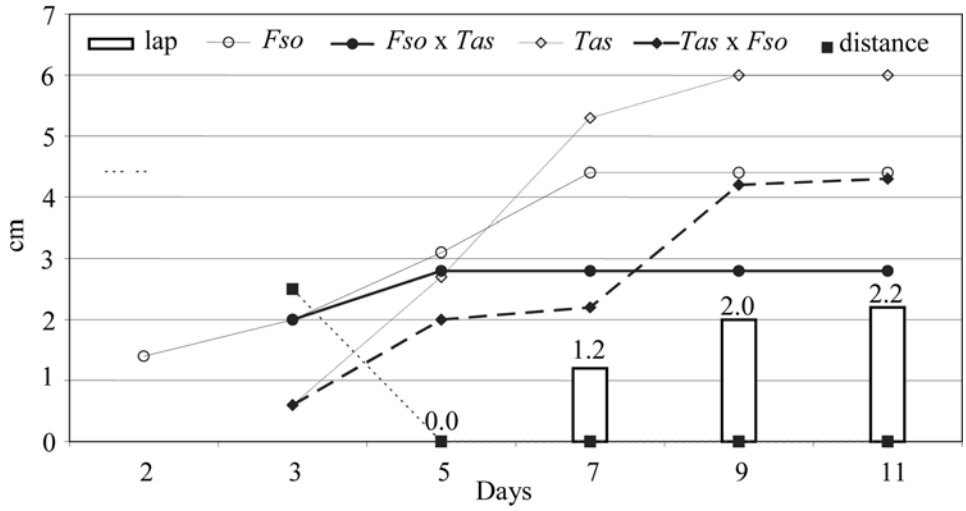

Fig. 11. *F. solani* isolate – F 5/IV belo

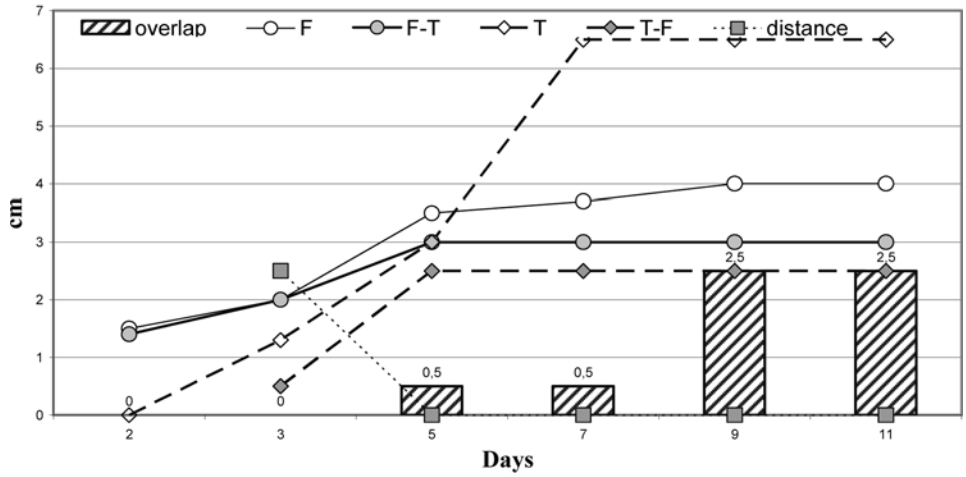

Fig. 9, 10, 11 — Mutual effects of *Trichoderma asperellum* (Tas) and *Fusarium solani* (Fso) isolates in dual culture; presented as radial mycelia growth (cm); growth of *F. solani* confronted with *T. asperellum* (Fso x Tas); growth of *T. asperellum* confronted with *F. solani* (Tas x Fso); black squares present distances between colony edges; columns present colonies overlap in cm.

Fig. 12. *Fusarium proliferatum* isolate F 8/I

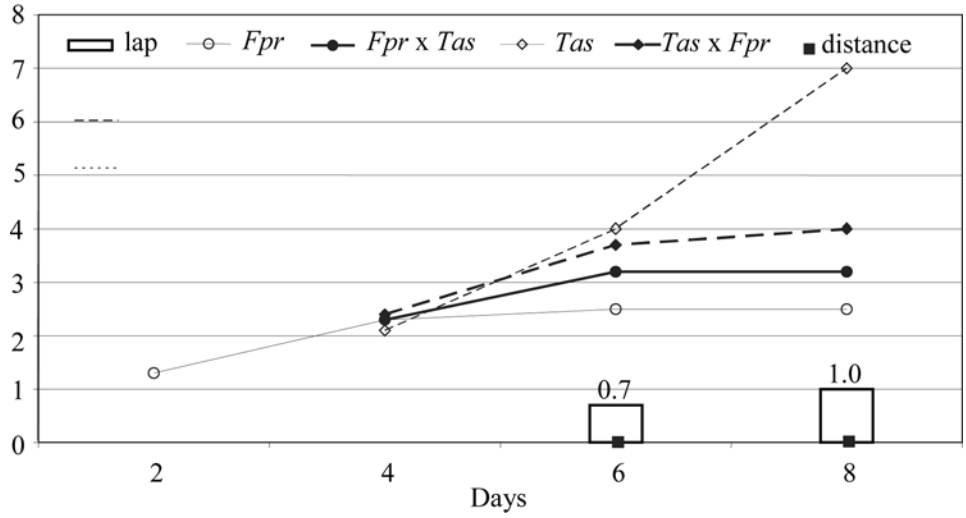

Fig. 13. *Fusarium proliferatum* isolate F K-III dance

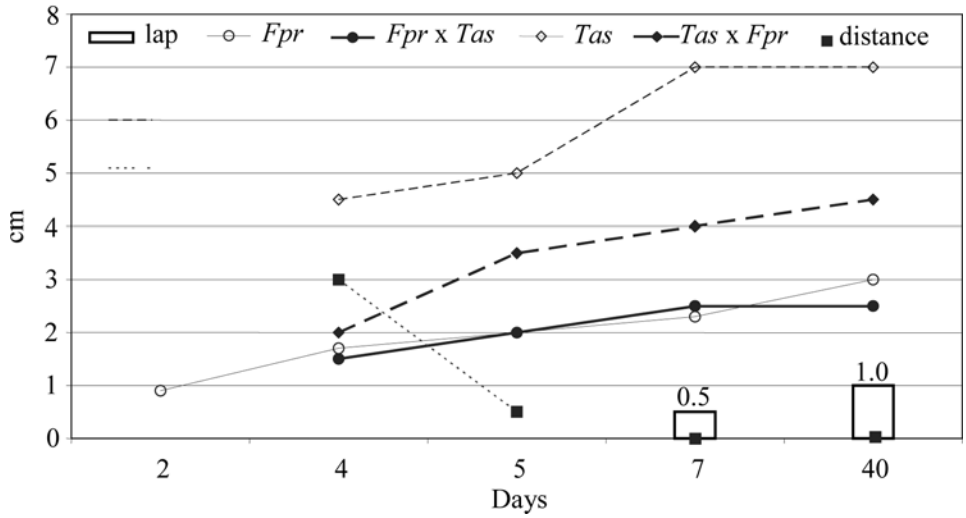

Fig. 14. *Fusarium proliferatum* isolate F 3-III B

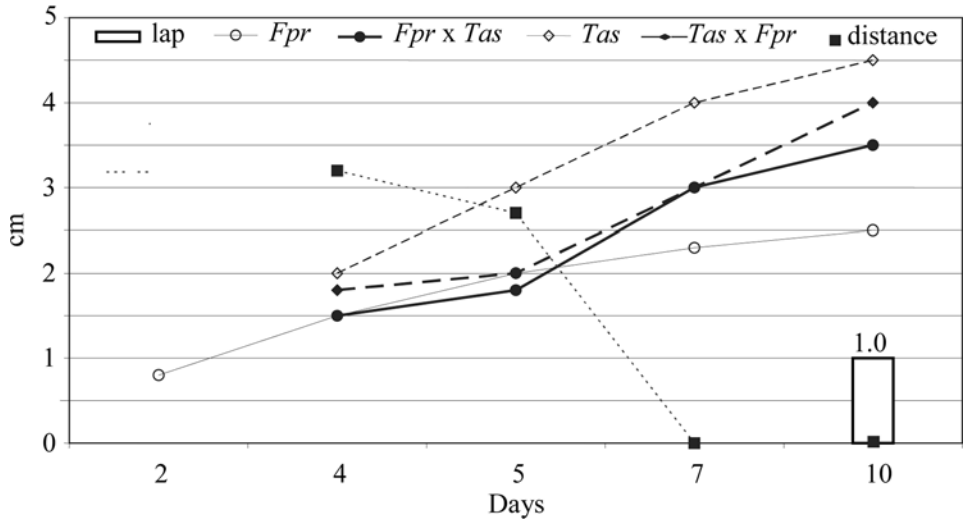

Fig. 12, 13, 14 — Mutual effects of *Trichoderma asperellum* (Tas) and *Fusarium proliferatum* (Fpr) isolates in dual culture; presented as radial mycelia growth (cm); growth of *F. proliferatum* confronted with *T. asperellum* (Fpr x Tas); growth of *T. asperellum* confronted with *F proliferatum* (Tas x Fpr).; black squares present distances between colony edges; columns present colonies overlap in cm.

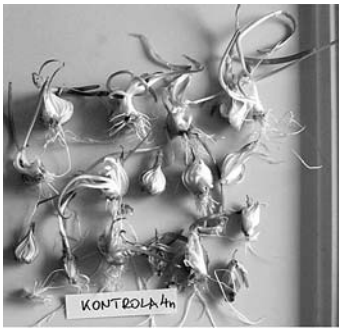

Fig. 15 — Control — non infected onion sets in pathogenicity test

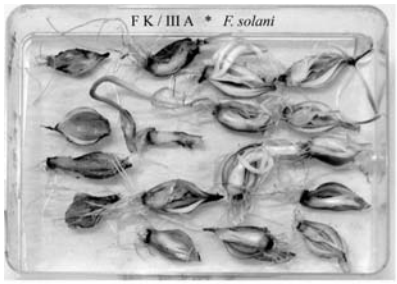

Fig. 16,

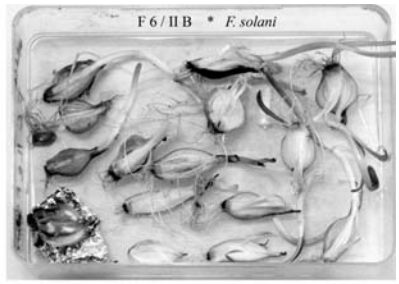

Fig. 17.

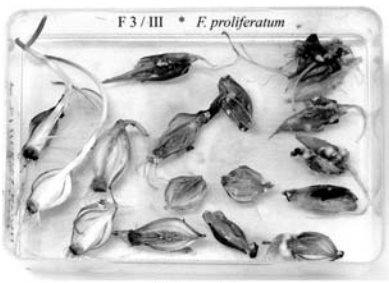

Fig. 18.

Fig. 19.

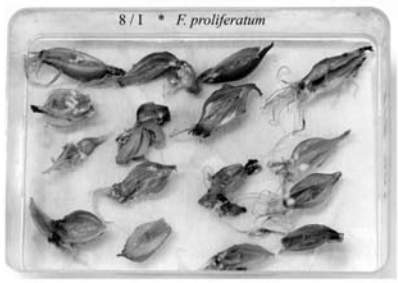

Fig. 20.

Fig. 16—20 — Onion sets artificially infected with different isolates of *F. solani* (16, 17) and *F. proliferatum* (18, 19, 20)

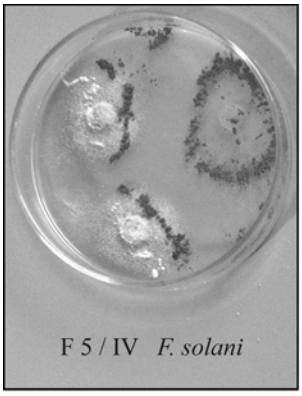

Fig. 21.

Fig. 22.

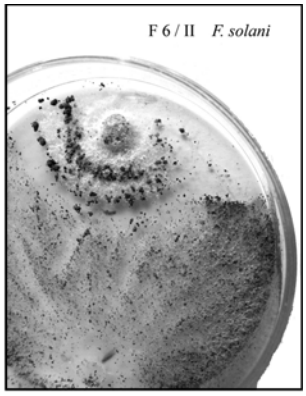

Fig. 23.

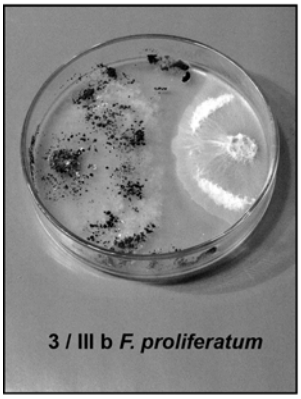

Fig. 24.

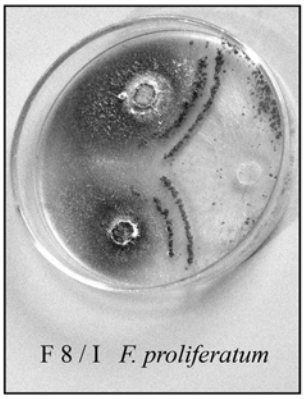

Fig. 25.

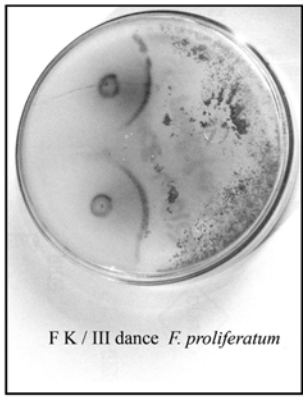

Fig. 26.

Fig. 21—26: Antagonism testing on solid media PDA: *F. solani* challenged by *T: asperellum* (21, 22, 23); *F. proliferatum* challenged by *T. asperellum* (24, 25, 26)

ФУЗАРИОЗНА ТРУЛЕЖ ЛУКА И МОГУЋНОСТ ПРИМЕНЕ БИОПРЕПАРАТА

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Резиме

Фузариозна трулеж лука најчешће је проузрокована врстама *Fusarium oxysporum* f. sp. *cepae* и *F. solani*, а и одскора и токсикогени *F. proliferatum* у нашим условима гајења и чувања лука у складиштима. Утврђена је највећа учесталост *F. proliferatum* и *F. solani* на луковицама из поља. Разлике изолата у патогености испољиле су се у различитом утицају на клијање лучица, издуживање клице и интензитет пропадања. Утврђена је различита антагонистичка активност *Trichodrema asperellum* на изолате *F. proliferatum* и *F. solani*, спорија инхибиција у случају првог и изражена у случају другог патогена у *in vitro* огледима. Антагонистичка својства врста из рода *Trichoderma* се искоришћавају за формулацију биолошких препарата примењивих у органској и конвенционалној производњи, у превенцији обољења лука која проузрокују земљишни паразити, пре свега фузариозног увенућа и трулежи. Истакнут је значај биолошких препарата у заштити здравствене безбедности произвођача и потрошача, с обзиром да су ови патогени потенцијални продуценти микотоксина.