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ЗА ПРИРОДНЕ НАУКЕ

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CONTENTS
САДРЖАЈ

ARTICLES AND TREATISES
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Зборник Матице српске за природне науке издаје Матица српска
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17-ACETALS OF 3-METHOXY-17-OXO-16,17--SECOESTRA-1,3,5(10)-TRIEN-16-NITRILE

ABSTRACT: The reaction of 3-methoxy-17-oxo-16,17-secoestra-l,3,5(10)-trien-16-nitrile (1) treated with mono- and dihydroxy alcohols has been studied with the aim of achieving optimal yields of 17-acetals. Best results have been obtained by the action of methanol and diethylene glycol on compound **1**, in the presence of *p*-toluene sulfonyl chloride as catalyst.

KEY WORDS: 16,17-seco estrone derivatives, acetal formation of.

INTRODUCTION

Within the framework of a broader project directed towards obtaining potential antiestrogens, 3-methoxy-17-oxo-16,17-secoestra-l,3,5(10)-trien-16-nitrile was synthesized [Miljković et al., 1990] as the key intermediate. Successive transformations of its aldehyde function resulted in a series of 16,17--seco-16-cyano derivatives [Petrović et al., 1977; Pejanović, 1991]. In biological tests *in vivo* most of them showed an almost total loss of estrogenic activity, whereas some representatives expressed a considerable antiestrogenic effect [Petrović et al., 1998].

It is well-known that the estrogenic, i.e., antiestrogenic, activity of estrone derivatives is strongly influenced by the nature of the substituents at C-17, as well as C-16 [Sakač, 1997]. Hence, it was interesting to try the synthesis of 16,17-seco-estrone derivatives bearing various functional groups at C-16, using the aldehyde **1** as starting compound.

The first step in this transformation involves protection of the aldehyde function of the starting compound. It is well known that the aldehyde group is most commonly protected by its transformation to acetals [Loewenthal,

* Corresponding author.

1973] in the reaction with alcohols. However, the yields of acetals can vary widely, depending on the nature of the aldehyde and alcohol.

Therefore, the aim of this paper was to study the formation of acetals of 3-methoxy-17-oxo-16,17-secoestra-l,3,5(10)-trien-16-nitrile (**1**) treated with mono- and dihydroxy alcohols, in order to establish conditions needed for obtaining highest yields of the protected derivatives.

EXPERIMENTAL

Infrared spectra (ν in cm^{-1}) were recorded as film on a Perkin-Elmer M457 spectrophotometer. NMR-spectra were taken on a Bruker AC 250E spectrometer and are reported in parts per million downfield from the tetramethylsylane internal standard; symbols s, bs, d, dd, q and m denote singlet, broad singlet, doublet, double doublet, quartet and multiplet, respectively. Mass spectra were recorded on a Finnigan-Math 8230 instrument, using electron impact (70eV) or chemical ionization (*iso*-butane) techniques; the first number denotes *m/z* value, and the ion abundances are given in parantheses.

*3,17,17-Trimethoxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile (**2**)*

3-Methoxy-17-oxo-16,17-secoestra-l,3,5(10)-trien-16-nitrile (**1**; 1 g, 3.37 mmol) was dissolved in methanol (20 cm^3) and *p*-toluene-sulfonic acid (60 mg) was added. The reaction mixture was kept at room temperature for 36 hrs, then poured into a saturated solution of sodium bicarbonate (150 cm^3) and extracted with chloroform (3x70 cm^3). The extract was washed with water, dried over anhydrous sodium sulfate and evaporated to dryness. The oily crude **2** (1.05 g, 90.91%) was chromatographed on a silica column (100 g silica gel; hexane-ethyl acetate /4:1/); the yield of pure pale oily 3,17,17-trimethoxy--16,17-secoestra-1,3,5(10)-trien-16-nitrile (**2**) was 0.82 g (71.00 %).

IR spectrum: 2937, 2867, 2834, 2241, 1610, 1578, 1504, 1466, 1430, 1387, 1288, 1258, 1240, 1162, 1104, 1072, 668.

^{1}H NMR spectrum ($CDCl_3$): 1.01 (s, 3H, C-18); 3.89 (s, 1H, C**H**$(OCH_3)_2$); 3.77 (s, 3H, OCH_3, C-3); 3.54 (d, 6H, OCH_3); 6.63 (d, 1H, H-4, J = 2.7 Hz); 6.70 (dd, 1H, H-2, J_1 = 2.8 Hz, J_2 = 8.6 Hz); 7.19 (d, 1H, H-1, J = 8.6 Hz).

^{13}C NMR spectrum ($CDCl_3$): 15.17 (CH_3, C-18); 16.16 (C-15); 55.09 (OCH_3); 111.02 (C-2); 113.35 (C-4); 120.32 (CN); 126.28 (C-1); 131.53 (C-5); 157.52 (C-3).

MS: 311(M^+-CH_3OH), 279, 239, 161.

*3-Methoxy-17,17-ethylendioxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile(**3**) and 3-methoxy-17,17-ethylendioxy-16,17-secoestra-1,3,5(10)-trien-16-carboxylic acid 16-ethylen glycol ester (**4**)*

Seco-cyanoaldehyde **1** (1 g, 3.37 mmol) was dissolved in ethylene glycol (20 cm^3) and *p*-toluene sulfonic acid (140 mg) was added. The reaction mix-

ture was refluxed for 7 hrs, then poured into a saturated solution of sodium bicarbonate (150 cm^3) and extracted with ether (3x70 cm^3). The extract was washed with water, dried over anhydrous sodium sulfate and evaporated in vacuum to dryness. The oily residue was chromatographed on a silica column (100 g silica gel; methylene chloride: ethyl acetate /10:1/), whereby two pure compounds were isolated: 3-methoxy-17,17-ethylendioxy-16,17-secoestra-l,3,5-(10)-trien-16-nitrile (**3**) and 3-methoxy-17,17-ethylendioxy-16,17-secoestra-1,3,5-(10)-trien-16-carboxylic acid 16-ethylen glycol ester (**4**).

Compound **3**, yield 300 mg (26.00 %), colorless oil.

IR spectrum: 2927, 2243, 1608, 1572, 1500, 1466, 1431, 1376, 1306, 1252, 1156, 1121, 1042, 868, 825, 809, 755, 668.

^{1}H NMR spectrum ($CDCl_3$): 1.11 (s, 3H, C-18); 2.48 (dd, 1H, H-15a, $J_{14,\ 15a}$ = 4.7 Hz); 2.60 (dd, 1H, H-15b, $J_{14,\ 15b}$ = 6.5 Hz, $J_{15a,\ 15b}$ = 17.2 Hz); 3.47 and 3.69 (2m, 4H, OCH_2CH_2O); 3.79 (s, 3H, OCH_3); 6.68—6.80 (m, 2H, H-2 and H-4); 7.16 (d, 1H, H-1).

^{13}C NMR spectrum ($CDCl_3$): 17.97 (C-15); 22.37 (CH_3, C-18); 42.17 (C-8); 55.26 (OCH_3); 61.76 and 72.56 (OCH_2CH_2O); 77.13 (C-17); 111.02 (C-2); 113.35 (C-4); 119.93 (CN); 123.46 (C-1); 158.43 (C-3).

MS spectrum: 341(M^+), 301, 251, 239, 226.

Compound **4**: yield 550 mg (40.00%), colorless oil.

IR spectrum: 3427, 2958, 2928, 1729, 1654, 1608, 1573, 1500, 1464, 1379, 1273, 1192, 1156, 1124, 1074, 1042, 958, 869, 812, 746.

^{1}H NMR spectrum ($CDCl_3$): 0.97 (s, 3H, C-18); 2.40 (dd, 1H, H-15a, $J_{14,\ 15a}$ = 4.3 Hz); 2.56 (dd, 1H, H-15b, $J_{14,\ 15b}$ = 7.5 Hz, $J_{15a,\ 15b}$ = 16.5 Hz); 3.48 and 370 (2m, 4H, OCH_2CH_2O); 3.79 (s, 3H, OCH_3); 3.83 (m, 2H, C**H**$_2$OH); 4.22 (m, 2H, CH_2OCO); 6.65—6.75 (m, 2H, H-2 and H-4); 7.12 (d, 1H, H-1).

^{13}C NMR spectrum ($CDCl_3$): 21.56 (CH_3, C-18); 36.51 (**C**H_2COO); 55.18 (OCH_3); 60.80 (CH_2OH); 61.66 and 72.71 (OCH_2CH_2O); 68.06 (COO**C**H_2); 77.20 (C-17); 110.87 (C-2); 113.26 (C-4); 122.98 (C-1); 126.13 (C-5); 137.10 (C-10); 158.06 (C-3); 174.19 (C = O).

MS spectrum: 404 (M^+), 343, 314, 288, 239, 226, 211.

*3-Methoxy-17,17-(1,4,7-trioxitetramethylene)-16,17-secoestra--1,3,5(10)-trien-16-nitrile(**5**)*

Compound **1** (1 g, 3.37 mmol) was dissolved in diethylene glycol (20 cm^3) and *p*-toluene sulfonic acid (40 mg) was added. The reaction mixture was refluxed for 5.5 hrs, then poured into a saturated solution of sodium bicarbonate (150 cm^3) and extracted with ether (3x70 cm^3). The extract was washed with water, dried over anhydrous sodium sulfate and evaporated in vacuum to dryness, yielding 1.32 g of oily crude product **5**. Flesh chromatography of the crude product (100 g silica gel 32—63 mesh, methylene chloride: ethyl acetate /10:1/) afforded 1.02 g (79%) of pure 3-methoxy-17,17-(1,4,7-trioksitetramethylene)-16,17-secoestra-1,3,5(10)-trien-16-nitrile (**5**) in the form of colorless oil.

IR spectrum: 3473, 2927, 2243, 1724, 1608, 1572, 1500, 1466, 1456, 1430, 1376, 1306, 1251, 1109, 1069, 1041, 756.

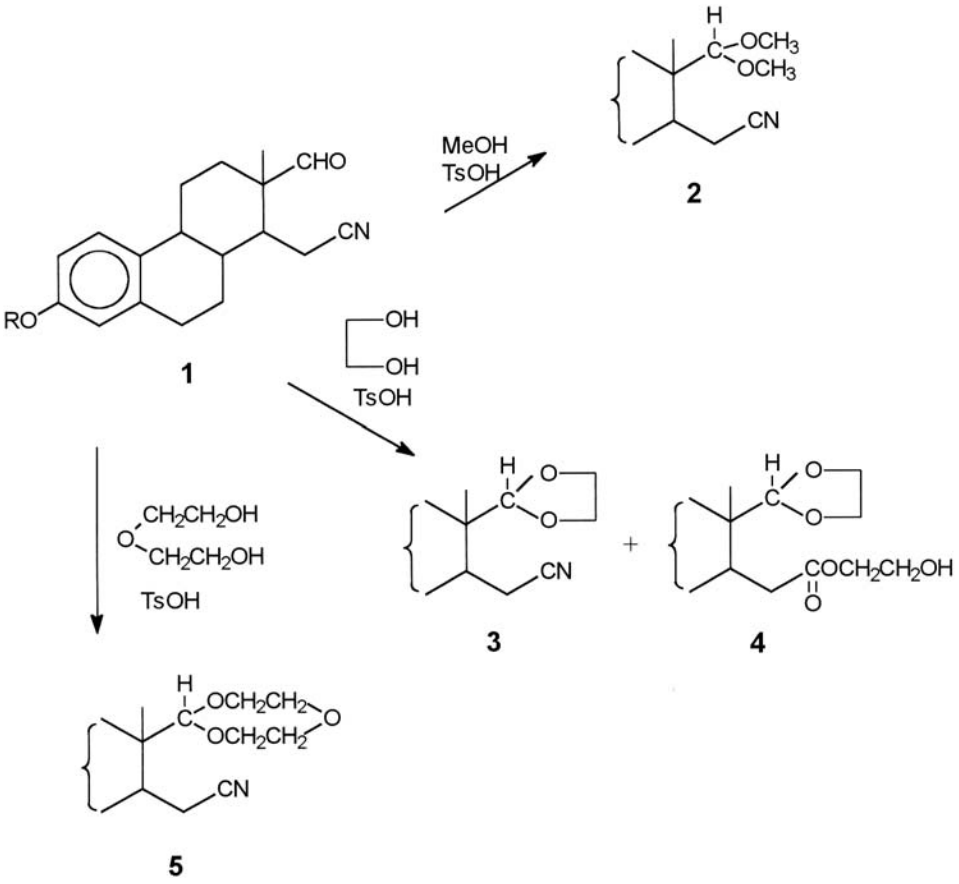
^{1}H NMR spectrum ($CDCl_3$): 1.14 (s, 3H, C-18); 3.79 (s, 3H, OCH_3); 6.68—6.8 (m, 2H, H-2 and H-4); 7.16 (d, 1H, H-1).

^{13}C NMR spectrum ($CDCl_3$): 55.26 (OCH_3); 61.76 and 72.56 (OCH_2CH_2O); 77.13 (C-17); 111.028 (C-2); 120.09 (CN); 113.40 (C-4); 123.52 (C-1); 129.87 (C-10); 137.45 (C-5); 158.46 (C-3).

MS spectrum: 385 (M^+), 345, 279, 251, 239, 225.

RESULTS AND DISCUSION

The starting compound, 3-methoxy-17-oxo-16,17-secoestra-1,3,5(10)-trien--16-nitrile (**1**), was obtained from 3-methoxy-17β-hydroxyestra-1,3,5(10)-trien--16-one oxime by the conventional procedure [Miljković et al., 1990]. The action of methanol upon compound **1** at room temperature, in the presence of catalytic amounts of *p*-toluene sulfonic acid afforded 3,17,17-trimethoxy-



-16,17-secoestra-1,3,5(10)-trien-16-nitrile (**2**) in 71% yield. Also, high yield of 3-methoxy-17,17-(1,4,7-trioxitetramethylene)-16,17-secoestra-1,3,5(10)-trien--16-nitrile (**5**) was obtained by refluxing the diethylene glycol solution of compound **1** in the presence of *p*-toluene sulfonic acid.

Surprisingly, in the acid-catalyzed reaction of compound **1** with ethylene glycol, the expected dioxolane **3** was formed as a minor product (26%), the main product (40%) being a more polar compound. On the basis of spectral data of the compound, structure **4** was ascribed to it.

Namely, in the IR spectrum, the bond for CN-function at 2243 cm^{-1} is absent, while an intensive new bond appears at 1729 cm^{-1} corresponding to the ester group vibrations. On the other hand, in the ^{1}H NMR spectrum, besides the signals for protons from the ethylene acetal function (—OCH_2CH_2O—) at 3.48 and 3.70 ppm, two additional multiplets at 3.83 ppm (—C**H**$_2$OH) and 4.22 ppm (—C**H**$_2$OCO—) can be observed. At the same time, the ^{13}C NMR spectrum of compound **4** shows no signal for the nitrile function at C-16, but three new signals appear at 60.80 ppm (—**C**H_2OH), 68.06 ppm (COO**C**H_2) and 174.19 ppm (C = O). Finally, the presence of the molecular ion at 404 mass units in the mass spectrum confirms the proposed structure.

The formation of compound **4** could be explained by acid-catalyzed saponification of the nitrile function in compound **3** (or **1**), followed by subsequent esterification with ethylene glycol.

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17-АЦЕТАЛИ 3-МЕТОКСИ-17-ОКСО-16,17--СЕКОЕСТРА-1,3,5(10)-ТРИЕН-16-НИТРИЛА

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Резиме

Проучавана је реакција 3-метокси-17-оксо-16,17-секоестра-1,3,5(10)-триен--16-нитрила (**1**) са моно- и дихидроксилним алкохолима, са циљем постизања оптималних приноса 17-ацетала. Најбољи резултати постигнути су дејством метанола и етиленгликола на једињење **1**, у присуству *p*-толуенсулфонске киселине као катализатора.

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HPLC DETERMINATION OF BIOGENIC AMINES IN SEA FISH AND THEIR CANNED PRODUCTS

ABSTRACT: A procedure is described for the extraction, extract purification by thin layer chromatography — membrane filtration and simultaneous determination of tyramine and histamine in sea fish and permanently canned fish using high performance liquid chromatography (HPLC).

Methanol was used to extract histamine and tyramine from homogenized fish meat. The extracts were purified by thin layer chromatography and the contents of these biogenic amines determined by HPLC. The contents of tyramine and histamine were below the officially acceptable tolerance limits in all the investigated fish samples.

KEY WORDS: histamine, tyramine, sea fish, can, HPLC

INTRODUCTION

Fish meat and its products as a source of high-quality, biologically important proteins should occupy a more important place in daily and dietary nutrition of people. The basic value of this food stems from its favorable chemical composition and content of essential fatty acids and amino acids. Fish meat contains 75—85% of water, 0.5—20% of fats, 15—24% of proteins, whereas carbohydrates are practically absent. Essential amino acids [Jevtić et al., 1979], essential polyunsaturated fatty acids [Vujković et al., 1991], liposoluble vitamins, minerals elements (Ca, Mg, P), as well as other important oligoelements are the reasons for a regular use of this food in the nutrition of the world's population.

Apart from all positive characteristics, fish meat has a negative one, perishability. The fish surface is covered by microflora containing also proteolytic bacteria. From the surface, even when minimal damages occur in the course of technological processing, these bacteria can easily reach the subcutaneous tissue and cause proteolytic degradation of amino acids. Depending on the original amino acid, various amines are formed by the decarboxylation process. Most frequently these are histamine formed from histidine, tyramine

from tyrosine, tryptamine from tryptophan, fenylethylamine from fenylalanine, cadaverin from lysine, putrescin from omithine. The presence of these biogenic amines, known in the literature as „volatile bases", in fish and fish products indicates the degree of chemical contamination of the fish. High concentrations of histamine in food are important from the toxicological point of view because they cause histaminic poisoning such as scombroid toxicosis. Study of the occurrence of amines in food is interesting from both the hygienic and toxicological aspects. The biogenic amines in fish cans are indicators of the degree of microbiological contamination, low technological quality of the product, and also of failures in the processing technology.

In view of the technological and hygienic importance of histamine and tyramine as biogenic amines, numerous authors have studied the possibility of their determination. The literature mentions a number of methods for identification an/or determination of these amines in fish products, e.g., thin-layer chromatography (TLC) [Lieber et al., 1978], gel electrophoresis [Rubach et al., 1981], spectrophotometry [Teodorović et al., 1995], high performance liquid chromatography (HPLC) with fluorescent or electrochemical detection [Subden et al. 1978; Skofitsch et al. 1981], gas chromatography, either alone or coupled with mass spectrometry [Henion et al., 1981].

In the present work we studied the possibility of applying a modified procedure consisting of the extraction, TLC and membrane filtration of the extract purification, and finally simultaneous HPLC determination of histamine and tyramine in sea fish and fish cans taken by random choice sampling from the market.

MATERIAL AND METHODS

The method of standard addition was used for the determination of the detection limit and the repeatability of the extraction and the chromatographic procedure. To prepare an extract with standard addition, reference standards of histamine and tyramine in an amount of 0.5 mg/10 g fish meat were added to the weighed fish samples for which a low content of analyte had been established.

Separation of histamine and tyramine from the model systems and investigated canned fish samples was carried out by double extraction with methanol. Samples of 10 g of fish were macerated with a twofold volume of methanol while heating the homogenate at 60°C in a water bath for 15 minutes. Samples were cooled quickly in a freezer, and then the volume adjusted to the initial value by adding methanol. The content was filtered through the Whatman No. 1 filtering paper and then centrifuged at 6,000 rpm for 15 minutes.

Purification of extracts was carried out by TLC on silica gel 60G using a mixture acetone — benzene — methanol — 25% ammonia (40:35:20:5 v/v/v/v). Chromatographic spots were identified with ninhydrin reagent followed by heating of the developed chromatograms in an oven at 80°C. To obtain a stable color of amine with copper, spraying with a copper sulphate solution, also followed by heating, may be used as an alternative procedure.

In the determination of the concentrations of histamine and tyramine by HPLC, the part of the thin layer on which the samples were separated was not sprayed with ninhydrin reagent. After comparing the R_f values with those for histamine and tyramine, the thin layer patches where these biogenic amines were expected to be located were mechanically stripped and dissolved in methanol, and the adsorbent layer was removed by centrifugation. The obtained methanolic extract was filtered through a membrane filter (pore size 0.22 μm) and, after adjusting to a volume of 1.00 ml, analyzed by the HPLC method.

To construct the calibration graph 5 reference standard solutions of histamine and tyramine in methanol were injected, so that tyramine content was in the range of 0.20—1.00 μg, and histamine of 0.10—0.70 μg.

This analytical procedure was employed to investigate 19 samples of canned sea fish taken from the market by random choice method, and the samples originated from different continents: 9 from Europe, 4 from Asia, 3 from Africa, 2 from South America and 1 from North America.

The mobile phase in the HPLC determinations was a buffer solution containing an organic modifier (15% methanol) and 0.01 mol dm^{-3} solution of KH_2PO_4 in water. Under the chromatographic conditions employed, at a wavelength of 215 nm, it was possible to determine simultaneously histamine and tyramine but also other biogenic amines and their precursor amino acids. At a flow rate of the mobile phase of 1.2 ml min^{-1} on the reversed-phase column Bio Sil C-18 HL (250x4 mm), the retention time for tyramine was 13.38 and for histamine 16.96 min. The data were treated using the software package which is a part of the chromatographic system employed.

RESULTS AND DISCUSSION

The extraction yield for the fish samples with standard addition of histamine and tyramine in three replicates was 91.9; 92.8, and 95.0% for tyramine and 99.5, 101.1, and 102.1% for histamine, which is in good agreement with the literature data [Teodorović et al., 1995; Tokvić and Slavić 1986]. The detection limit of the applied HPLC determination was 0.025 μg for histamine in a probe and 0.02 mg of histamine in 100 g of fish, the values for tyramine being 0.05 μg in a probe and 0.05 mg in 100 g of fish.

According to the regulations (Regulations concerning the amounts of pesticides, …, 1992) the allowed content of amines (referring solely to histamine) in canned fish must not exceed 40 mg/100 g. Using the described procedure of extraction, purification and HPLC determination, it is possible to determine histamine in concentration that is about 200 times lower than the maximal tolerable value given by the Regulations.

Figure 1 presents a typical chromatogram of a standard mixture of histamine and tyramine and Figures 2 and 3 give the calibration graphs for the determination of histamine and tyramine, respectively.

Table 1 presents the results of the determination of histamine and tyramine in the investigated fish can samples.

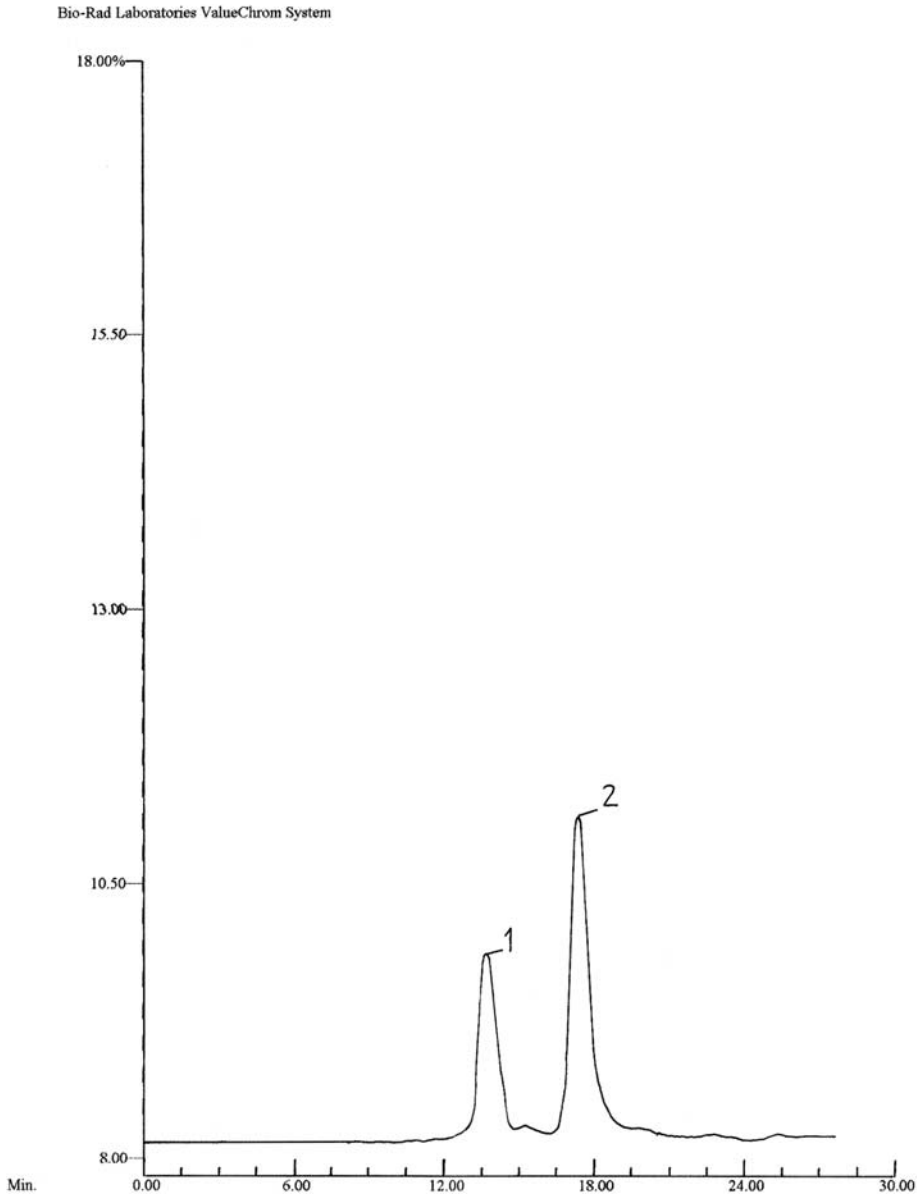

Fig. 1 — Typical chromatogram of the standard mixture of histamine and tyramine (1 — tyramine; 2 — histamine)

It is evident from the table that only one sample contained histamine in the amount of 9.564 mg/100 g, and for 9 samples the histamine content was about 2 mg/100 g, which is about 20 times lower than the tolerable concentration mentioned in the Regulations. In the remaining 9 samples, the content was up to 30 times lower than the corresponding tolerance limit.

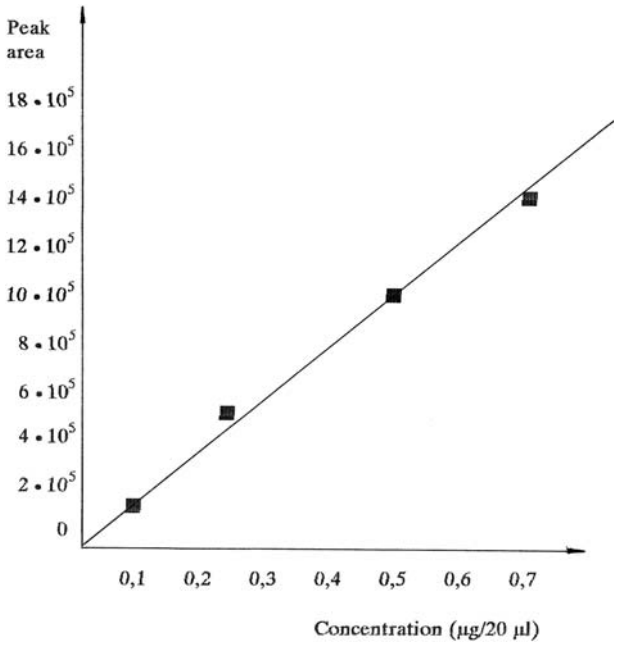

Fig. 2 — Calibration graph for the determination of histamine

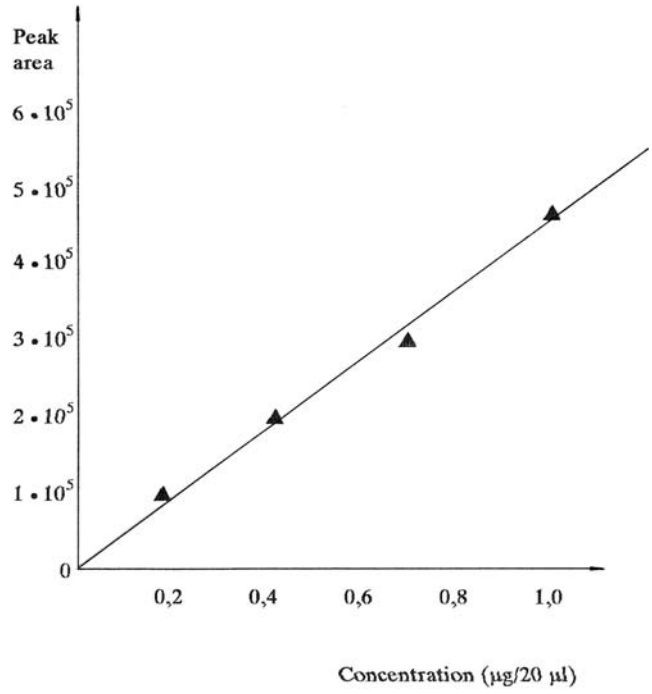

Fig. 3 — Calibration graph for the determination of tyramine

Tab. 1 — Contents of biogenic amines in canned fish samples

Name	Country of origin	Content (mg/100 g)	
		Tyramine*	Histamine
Universal-sogenco	Morocco	0.810	1.233
Arthur	Canada	0.913	0.884
Lafit	Greece	0.913	1.117
La peria	Philippine	0.822	1.328
Stella maris	Slovenia	0.799	1.998
Prima kost	USSR	0.866	9.564
Slavianka	Bulgaria	0.916	1.804
Ropotamo	Bulgaria	0.699	2.024
Oriente	Venezuela	0.996	2.066
Marka Beograd	Yugoslavia	0.776	1.065
Topp fisch	Thailand	0.900	1.989
Diamond	Thailand	0.782	0.965
Hunter	Thailand	0.855	1.651
Belamar	Portugal	0.921	1.429
Manu	Morocco	0.874	2.116
Marcella (calamare)	Greece	0.877	2.555
Marcella (octopus)	Greece	0.798	1.943
Zimfjad	Denmark	0.821	1.134
Mayona	Holland	0.995	2.033

* The tollerance limit not prescribed by the Regulations

CONCLUSION

a) By determining histamine and tyramine in model systems with the efficiency of the analytical procedure in a range from 91.9 to 102.1%, the applicability of the modified analytical procedure was proved and validated in the laboratory of the Scientific Veterinary Institute „Novi Sad".

b) The method described is applicable for a fast determination of tyramine and histamine in sea fish and canned sea fish. Also, under somewhat modified extraction conditions, the procedure can be applied for the determination of other biogenic amines in other foodstuffs (cheese, chocolate, dairy products, sauerkraut).

c) Of the 19 canned sea fish samples investigated, only one had an increased content of histamine, which was still 4 times lower than the tolerable concentration allowed by official regulations.

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ТЕЧНО-ХРОМАТОГРАФСКО ОДРЕЂИВАЊЕ БИОГЕНИХ АМИНА У МОРСКИМ РИБАМА И ЊИХОВИМ КОНЗЕРВАМА

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Резиме

У раду је описан поступак екстракције и пречишћавања екстраката системом хроматографија на танком слоју — мембранска филтрација за истовремено одређивање тирамина и хистамина у риби и трајним рибљим конзервама, применом течне хроматографије под високим притиском.

Из хомогенизованог рибљег меса хистамин и тирамин екстрахују се метанолом. Екстракт се пречишћава применом хроматографије на танком слоју, а за одређивање садржаја ових биогених амина у испитиваним узорцима примењује се течна хроматографија под високим притиском. У свим испитиваним узорцима рибљих конзерви садржај тирамина и хистамина био је испод максимално дозвољене концентрације прописане *Правилником*.

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MATHEMATICAL MODELING OF THE DELAYED FLUORESCENCE INDUCTION CURVE COMPONENTS

ABSTRACT: The present study deals with mathematical modeling of the components (phases, bands) obtained by resolution of the delayed fluorescence (DF) induction curve. The components are in certain connection with electrochemical gradient (ECG) formed across the thylakoid membranes upon illumination. The fitting of the *C* and *D* components (bands) by using a consecutive model for first-order reactions (A → B → C) shows that they might play a role of an intermediate (B), according to the following:

$$(\text{„}A_1\ state\text{"})_{ECG} \xrightarrow{k_1\,(C\ band)} C\ band \xrightarrow{k_2\,(C\ band)} products$$

$$(\text{„}A_2\ state\text{"})_{ECG} \xrightarrow{k_1\,(D\ band)} D\ band \xrightarrow{k_2\,(D\ band)} products$$

The two ECG controlled „states" (A_1 and A_2) are not identical, which does not exclude some sort of proportionality. On the other hand, the *E* component, that contributes mainly to the stationary level of delayed fluorescence induction curve, obeys the parallel model of at least two first order reactions.

KEYWORDS: delayed fluorescence, DF induction curve, mathematical modeling.

INTRODUCTION

The delayed fluorescence (DF) phenomenon can be described as the glowing of green plants, algae and photosynthetic bacteria in the red range of the visible spectrum subsequently to illumination. In its final step DF is formed by the same S_1 — S_0 transition as prompt fluorescence [Lang et al., 1991; Krause et al., 1991]. But the very different lifetimes, of 1.5 ns or less for prompt fluorescence [Govindjee et al., 1990; Schmuck et al., 1992] compared with nanoseconds [Sonneveld et al., 1981], over microseconds [Haveman et al., 1975; Holzapfel et al., 1974] and milliseconds [Hipkins et al., 1974; Barber et al., 1974] to seconds range [Rutherford et

al., 1984] for DF, clearly point to two very distinct mechanisms by which photo-active S_1 state of chlorophyll (Chl) is created. In the case of prompt fluorescence, the S_1 state is formed by absorption of a proton. In the DF's case the S_1 state is created through recombination of products formed in the primary photochemical act [Govindjee and Papageorgiou, 1971; Papageorgiou et al., 1975; Jursinić et al., 1986]. Thus, unlike prompt fluorescence, the entire entity of photosynthetic apparatus determines the DF emission properties.

The delayed fluorescence induction curve reflects processes and phenomena that take place when a photosynthetic object is being kept in dark for a while, and then illuminated, *i.e.*, in a transition period, from the „dark" to the „light" regimen. Most of DF induction curves have been recorded using a rotating disc which provides intermittent illumination (a few milliseconds of a light period and subsequently a few milliseconds of darkness in which DF is being recorded) [Vučinić, 1983; Marković et al., 1987]. The overall shape of the DF induction curve is highly dependent on the length of the dark period preceding illumination [Dzhibladze et al., 1988; Bukhov et al., 1989]. If the preceding dark period (τ) is longer than 30 s and shorter than 300 s, the DF induction curve is split into at least three phases [Radenović et al., 1985]. The clearly distinct appearance times of their maxima ($t_B = 5 \pm 0.5$ s; $t_C = 15 \pm 5$ s, and $t_D = 300 \pm 60$ s) suggest that they originate from distinct processes that take place during the dark/light transition period. Recently, Veselovsky [Veselovsky et al., 1990] made a step toward to the explanation of DF induction curve components by putting it on the same time scale with temporal transients of prompt fluorescence during continuous illumination of photo-synthetic apparatus (the so-called Kautsky effect), and with oxygen evolution changes. The Kautsky effect has been thoroughly investigated and reasonably well understood [Govindjee and Papageorgiou, 1971; Papageorgiou et al., 1975; Lichtenthaler and Rinderle, 1988; Lichtenthaler, 1992]. The comparison revealed a correlation between components (*B* and *C*) and the electrochemical gradient (ECG) formed across thylakoid membranes upon illumination [Veselovsky et al., 1990]. This study presents a further investigation of the correlation between DF induction curve and ECG by the use of mathematical models.

EXPERIMENTAL DETAILS

Two different maize genotypes were used in these experiments. The leaf segments (2,0 cm^2) were cut under water and placed on a temperature-controlled plate inside a phosphoroscope; they were adapted to the temperature of the plate (two different temperatures: 22°C and 32°C) and darkness (two different periods, τ = 210 s and 240 s), and then delayed fluorescence was monitored. The DF intensity was measured in the dark period of intermittently illuminated leaves, using a Becquerel phosphoroscope and a 150 W quartz-halogen lamp. One cycle consisted of 2 ms of light and 8 ms of darkness. Delayed fluorescence was registered by a cooled photomultiplier from the 3rd to 7th ms of the

dark period. The signal from the multiplier was monitored on a storage oscilloscope for the fastest processes, while the slower variation of DF was recorded on a chart. The few minutes recording produced the so-called DF induction curve, with faster transients in the first 2 minutes and a slower changes afterwards (Figs. 1a—2a). Details of the experimental setup have been already presented [V u č i n i ć, 1983].

Mathematical modeling

Decomposition of DF induction curves shown at Figures 1a & 2a was done by numerical methods. Mathematical modeling of the components (phases, bands) has been done by using a consecutive model of two first-order reactions (applied on *C* and *D* components) — Eq. (1) and a parallel model of two first-order reactions (applied on *E* component) — Eq. (4). In order to analyze, model and optimize each step of the process expressed by the components particularly, dynamical programming methods were used.

Concentration changes of participants involved in consecutive chain of the two firstorder reactions:

(1) $$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

are being expressed by the formulas:

(2) $$(B) \quad A_0 \frac{k_1}{k_2 \quad k_1} \exp(\ k_1 \ t) \quad \exp(\ k_2 \ t)$$

and:

(3) $$(C) \quad A_0 \quad 1 \quad \frac{1}{k_1 \quad k_2} \quad k_2 \ \exp(k_1 \ t) \quad k_1 \ \exp(\ k_2 \ t)$$

where k_1 and k_2 represent the two first-order rate constants and (A_0) is the initial concentration of primary reactant A.

Concentration changes of the participant M, involved in the two parallel first-order reactions:

(4) $$M \xrightarrow{q_1} N$$
$$M \xrightarrow{q_2} P$$

can be expressed as:

(5) $$(M) \quad (M_0) \ \exp(\ k_5 \ t)$$

where (M) and (M_0) are the time-dependent and initial concentrations of the reactant M, respectively, and $k_5 = q_1+q_2$, the sum of the two first-order rate constants.

The three chosen components (*C, D* and *E*) were fitted by the corresponding models (Eqs. 1 & 4, and their corresponding equations) by using the χ^2 — criterion of optimality, the statistic weights being equal to 1.

$$\chi^2 \quad \frac{\sum_{i=1}^{n} (y_{exp} - y_{model})^2}{n - m} \quad \frac{\sum_{i=1}^{n} y_i - y(x_i, k_1, k_2, \ldots, k_m)^2}{n - m} \quad \min \tag{6}$$

where variable *y* corresponds to variables (B) and (C) in Eq. (2) & (3), respectively, and to variable (M) in Eq. (5).

Non-linear model parameters were solved by using a system of equations:

$$(\chi^2)/ k_i, \qquad (i = 1, 2, \ldots m) \tag{7}$$

Optimum values of the model parameters were determined through the „Microcal Origin 3.5" program. For a chosen confidence level the program generates parameter values of optimal fitting (χ^2 — value, k_i coefficients and their standard errors, correlation coefficients etc.), shown at the bottom of Figures 1—2.

Validity of the models and confidence coefficients was checked for the confidence level of $\alpha = 0.05$. Validity of the models was confirmed by the Fischer F-criterion. As an additional confirmation criterion for the validity, correlation coefficient with the corresponding T-test was used. The confirmation of confidence coefficients was obtained by the analysis of error limits for the coefficients.

RESULTS AND DISCUSSION

The typical DF resolved induction curve is shown in Figures 1a—2a: the resolved *B, C, D* and *E* components are indicated under the trace. Figure 3 shows the *A* component recorded on the oscilloscope only, on the three expanded time scales. At the end of the induction part of the signal, stationary level is established after 2—3 minutes at room temperature [Dzhanumov et al., 1986; Klimov, 1988].

The component *A* is not influenced by any length of the τ period. Generally it is being considered to be tightly connected to the primary photochemical act in the PSII reaction center (RC) [Lavorel et al, 1975; Malkin et al, 1979].

There is an important agreement about a close correlation between the *B, C* and *D* components origin and the electrochemical gradient (ECG) formed across thylakoid membrane upon illumination. It was proven experimentally that DF emission obtained under rotating disc millisecond working regimen (which „produces" DF induction curve) exponentially depends on ECG [Crofts et al., 1971; Lavorel et al., 1982].

A simple interpretation of Hipkins—Barber [Hipkins and Barber, 1974] equation:

(8) $$J \quad (Z\ )(Q\ )\nu k' exp\ (\ E_a \quad \Delta p) / k_B T$$

yields the same conclusion. In the equation, J is the rate of the S_1 singlet state formation through recombination process, (Z^+) and (Q^-) are oxidized donor's and reduced acceptor's concentrations, respectively, ν is frequency factor, k' and k_B are the entropy term constant and the Boltzmann's constant, respectively; E_a is an activation energy for the recombination process that yields DF emission, and Δp is in fact ECG, being equal to:

(9) $$\Delta p \quad \Delta \Psi \quad (2.3\ RT/F)\ \Delta pH$$

with its electrical ($\Delta\Psi$) and ΔpH component. Additionally, Veselovsky and Veselova [Veselovsky and Veselova, 1990] offered a broader explanation about the ECG influence on the *C* and *D* components behavior. In their interpretation, the *C* component is controlled by the electrical ($\Delta\psi$) component of ECG, and the *D* component by the ΔpH-induced microstructural changes. Indeed, the behavior of the *B* and *D* components in the presence of compounds enabling ECG (*uncouplers*) „dissipation" confirmed such conclusions [Wraight and Crofts, 1971; Itoh et al., 1973; Lavorel et al., 1975]. The component *C* has a similar character, since its behavior is sustible to the presence of the uncoupler valynomicine [Veselovsky and Veselova, 1990].

The previous report on the DF induction curve kinetic behavior [Radenović et al., 1985] shows that the *C* and *D* components maxima dependence on the length of the preceding dark period (τ) may be expressed with two first-order consecutive reactions of different rate constants. This proves that their origin is in different processes that take place during dark/light transition period. One of the consequences of keeping the photosynthetic object (in this case, the leaf segment) in the dark is „disenergisation" of a thylakoid membrane, *i.e.* „dissipation" (disappearance) of ECG [Evans et al., 1973; Morita et al., 1981]. Since the proceeding dark period (τ) is a quantitative measure of the ECG dissipation, it was reasonable to try to apply a kinetic model for consecutive first-order reactions on the whole temporal behavior of the *C* and *D* components (rise, maximum and decline). Precisely, the goal was to check out how well the *C* and *D* components (obtained by theoretical resolution of the experimental DF induction curves (Figures 1a & 2a) fit the consecutive first--order reaction model. To do this, the consecutive model was applied to „digitized" *C* and *D* components (according to the procedure described in „Experimental details"), and then the simulated *C* and *D* components were compared with the experimental ones (*i.e.*, with those obtained by theoretical resolution of the experimental curves) (Figures 1b—2b).

Concentration changes of the participants involved in the consecutive chain of two first-order reactions (Eq. 1) are shown by Eqs. (2—3). For our purpose, A is considered as an ECG-controlled „state" whose temporal changes may cause a possible appearance of DF induction curve components. The term „state" is being based on all current up-dated knowledge about mechanisms that produce DF emission. Both today's accepted theories on the origin

of the DF emission (recombination and radical-pair theory) imply a creation of the chlorophyll S_1 photoactive state as a result of a series of consecutive back reactions and processes, with many participants at the donor, as well as at the PSII RC acceptor side [Jursinić et al., 1986]. Initial concentrations, as well as the rate constants for this whole reverse process may only be speculated: the experimental data have not been reported yet. A part of the process is undoubtedly ECG controlled, since the PSII donor and acceptor side lay on two sides of thylakoid membrane through which ECG is being established immediately upon illumination. This part of the whole reverse process might be considered as a „state" controlled by ECG, which is undoubtedly connected with the *C* and *D* components' existence [Veselovsky and Veselova, 1990]. Following this logic, it is even more logical that „the state" is being dissipated in first-order processes with the „rate constants" k_1 and k_2. Quotation marks have been used because k_1, as well as k_2 are not real rate constants, but rather „pseudo-constants" and, most probably, very complex ones.

Figures 1a & 2a show two different DF induction curves, obtained experimentally from two various maize leaf segments. Both curves were theoretically resolved into four components: *B, C, D* and *E.* The last one (without the peak) contributes mainly to the stationary level, established at the end of all DF induction curve changes. The separated *C* and *D* components have been put together with their theoretical models (as described in „Experimental details") according to Eq. (2) and presented at Figures 1b & 2b, respectively.

First of all, it is necessary to emphasize that it was *not* possible to model both the *C* and *D* the component (from both Figures 1a & 2a) according to Eq. (3). Two important conclusions could be drawn from this fact. First, the consecutive chain:

$$\text{„A state"} \quad \rightarrow \text{C component} \quad \rightarrow \text{D component}$$

simply does *not* exist. Its existence might be probably expected from the chronological point of view (the *C* component appears first, and the *D* component later — Figs. 1a & 2a), but, other than that, there is no further evidence to support the expectation. Second, the ECG-controlled „A state" is *not* the same for the *C* and *D* components. The additional proof for the last conclusion came from mutual comparison of k_1 and k_2 pseudo-constants, used for the modeling of *C* and *D* components, from the same experimental induction curve — Figures 1(a—b) & 2(a—b). The following results from Fiure 1b:

$$k_1\,(C_{band}) / k_1\,(D_{band}) \quad 0.10153 / 0.01794 \quad 5.7$$

and

$$k_2\,(C_{band}) / k_2\,(D_{band}) \quad 0.13153 / 0.01866 \quad 7.1$$

The following results from Figure 2b:

$$k_1\,(C_{band}) / k_1\,(D_{band}) \quad 0.07602 / 0.01029 \quad 7.4$$

and

$$k_2\ (C_{band}) / k_2\ (D_{band}) \quad 0.07665 / 0.01044 \quad 7.3$$

If the ECG-controlled „A state" is considered to be the same for *C* and *D* components, then it is reasonable to expect k_1 and k_2 pseudo-constants to be very close. That is obviously not the case. But, the fact that the „A state" is not the initial common „state" for *C* and *D* components appearance, does not mean that the consecutive first-order kinetic model (represented by Eq. (1)) cannot be applied any longer. On the contrary, the k_1/k_2 relationship for *C* and *D* components, taken separately, from both, Figures 1b & 2b, confirms its validness. The following results from Figure 1b:

$$(k_1 / k_2)_{C\ band} \quad 0.10153 / 0.13153 \quad 0.8$$

and

$$(k_1 / k_2)_{D\ band} \quad 0.01794 / 0.01866 \quad 1.0$$

and from Figure 2b:

$$(k_1 / k_2)_{C\ band} \quad 0.07602 / 0.07665 \quad 1.0$$

and

$$(k_1 / k_2)_{D\ band} \quad 0.01029 / 0.01044 \quad 1.0$$

Thus, the k_1/ k_2 relationships were approximately equal for both components, obtained from two different experiments, from two various leaf segments (and from two different maize genotypes). This certainly was not a coincidence. It clearly appears that the consecutive chain (Eq (1)) is in effect, but the *C* and *D* components cannot be linked to its final product (C) but rather to the „intermediate" (B). So, taken just schematically, instead of the first mentioned scheme:

$$(\text{„}A\ state\text{"})_{ECG} \quad \xrightarrow{k_1} C\ band \quad \xrightarrow{k_2} D\ band$$

the other one seems to be more reasonable:

$$(\text{„}A_1\ state\text{"})_{ECG} \quad \xrightarrow{k_1(C\ band)} C\ band \quad \xrightarrow{k_2(C\ band)} products$$

$$(\text{„}A_2\ state\text{"})_{ECG} \quad \xrightarrow{k_1(D\ band)} D\ band \quad \xrightarrow{k_2(D\ band)} products$$

The last scheme does not mean necessarily any parallelism between the two „A states", but some sort of proportionality cannot be neglected. The above cited and very constant relationships, k_1(*C* band)/k_1(*D* band) and k_2(*C* band)/k_2(*D* band), calculated from the same figures (about 5.7 and 7 from Figure 1b, and about 7 from Figure 2b) do not challenge the proposed scheme. The fact that the *C* constants are much bigger than the *D* ones simply means

that *C* component appears earlier, and *D* component later, which, of course, cannot be denied (Figures 1a & 2a).

Considering the *E* „component" (Figures 1a & 2a) it is clearly evident that the consecutive first-order kinetic model — Eq. (1) cannot hold any longer: that is clear even from the shape. This component appears as a „precursor" of the DF induction curve stationary level, to which the three components (*B, C* and *D*) contribute, at least partly, in a parallel manner. To check this out we tried to model *E* „component" with the simplest model for parallel processes: two first-order parallel reactions — Eq. (4) and corresponding equation (5). Figure 4 presents the experimental *E* „component" together with the model, with the k_5 value of 0.01098. This time, k_5 is a pseudo-constant for the same reasons that k_1 and k_2 are pseudo-constants for *C* and *D* components.

From the last value it can be proven that q_1 and q_2 first-order pseudo-constants are different from their „counterparts", the *C* and *D* components pseudo-constants, k_1 and k_2:

$$(k_5 \quad q_1 \quad q_2)_{\text{E „band"}} \quad 0.01098 \quad (k_1 \quad k_2)_{\text{C band}} \quad 0.10153 \quad 0.13153 \quad 0.23$$

and

$$(k_5 \quad q_1 \quad q_2)_{\text{E „band"}} \quad 0.01098 \quad (k_1 \quad k_2)_{\text{D band}} \quad 0.01794 \quad 0.01866 \quad 0.037$$

Furthermore, it clearly means that the initial „M state" (the „precursor" of the *E* „component") is different from „A_1 and A_2 states" („precursors" of *C* and *D* components). Here, the term „M state" is even more meaningful than in the case of „A states". As the modelling shows, at least two various, parallel, probably ECG-dependent processes are responsible for the *E* „component" (and so for the stationary level) existence. So far, it might only be speculated about a connection between the „A states" („A_1" and „A_2") and the „M state". But, probably, if the connection exists, it occurs *via* ECG functioning.

It is worth to underline again that the all conclusions were made by using two really *different* DF induction curves. The differences come not only from variously chosen photosynthetic objects (two different leaf segments from two different maize plants), but from various experimental conditions too: (τ = 240 s, T = 22°C for the first DF induction curve; τ = 210 s, T = 32°C for the second one). Therefore, it seems for certain that despite the chosen objects and conditions, not a coincidental, but rather regular behavior was reflected, in the sequence of photosynthesis „light phase" connected with ECG.

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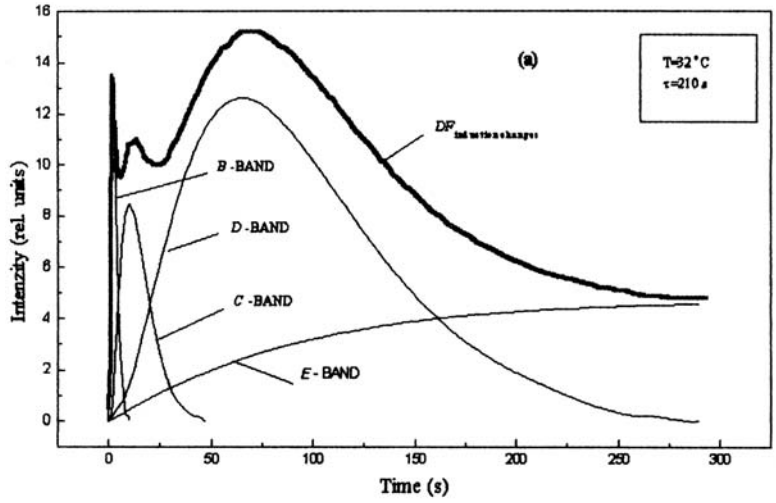
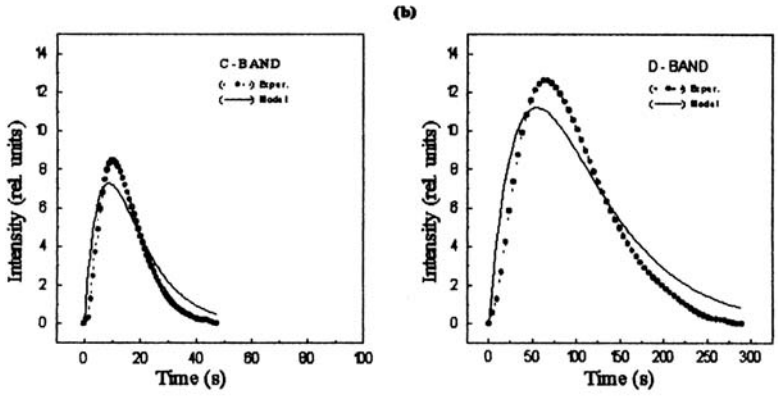
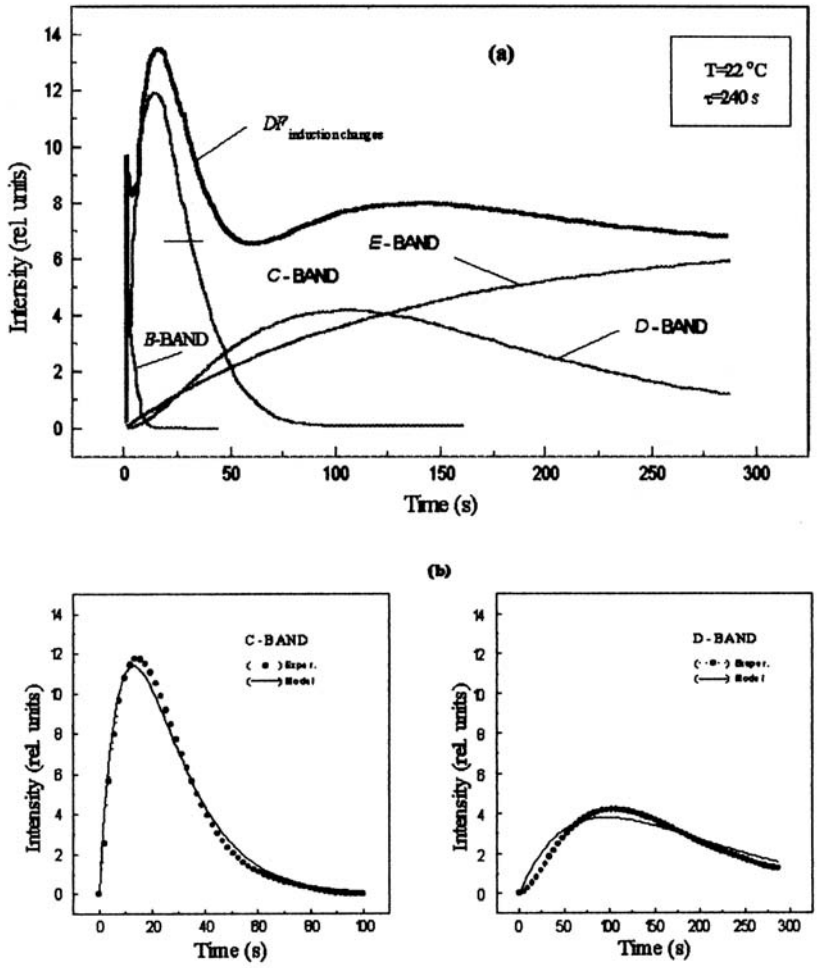




k1	0,10153	err	0,49982
k2	0,13153	err	0,50534
k3	2,29778	err	0,08242
χ^2			1,05394
Correlation			0,93967
Confidence			0,95
F=0,1914			
F_T=1,8363			
F<F_T			

k1	0,01794	err	0,06498
k2	0,01866	err	0,06858
k3	0,55268	err	0,04939
χ^2			1,8248
Correlation			0,95395
Confidence			0,95
F=0,1884			
F_T=1,3850			
F<F_T			

Fig. 1(a) — Delayed fluorescence induction curve and resolved bands: *B, C, D* and *E*. The signal was obtained as described in experimental part. Temperature of the leaf segment was 32°C, the preceding dark period (τ) was 210 *s*; (**b**) Mathematical modelling of the *C* band (left) and the *D* band (right), as described in experimental part, according to **Eq. (2)**. The bottom symbols' meaning: k_1, k_2 and k_3 (= $k_1(A_0)$) — pseudo-rate constants values with their errors (err); χ^2 — chi square; F — Fischer criterion; F_T — F — value taken from statistic tables for certain values of freedom degrees.



k1	0,07602	err	0,49982
k2	0,07665	err	0,50534
k3	2,37839	err	0,08242
χ^2			0,19226
Correlation			0,99451
Confidence			0,95
F=0,0605			
F_T=1,5330			
F<F_T			

k1	0,01029	err	0,16227
k2	0,01044	err	0,16451
k3	0,1064	err	0,00805
χ^2			0,15425
Correlation			0,94647
Confidence			0,95
F=0,1329			
F_T=1,5330			
F<F_T			

Fig. 2(a) — Delayed fluorescence induction curve and resolved bands: *B, C, D* and *E*. The signal was obtained as described in the experimental part. The leaf segment temperature was 22°C, the preceding dark period (τ) was 240 *s*; (**b**) Mathematical modelling of the *C* band (left) and the *D* band (right), as described in the experimental part, according to **Eq. (2)**. The meaning of bottom symbols is the same as for **Fig. 1b**.

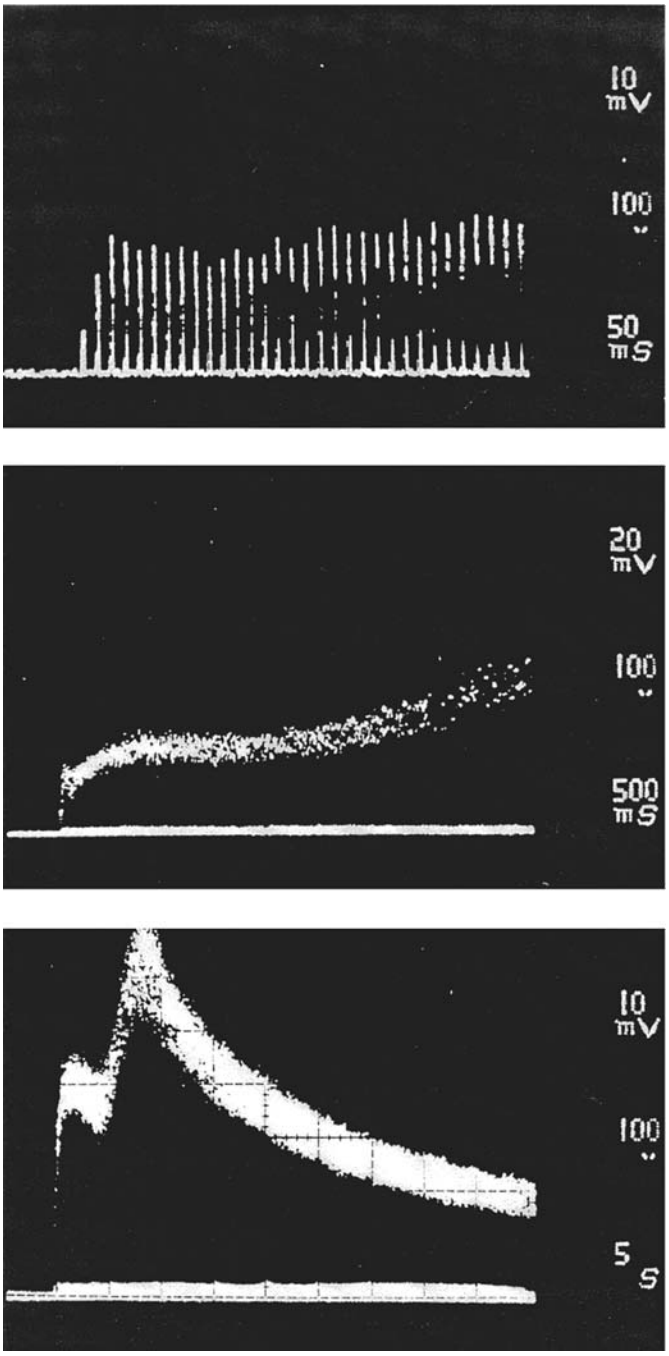
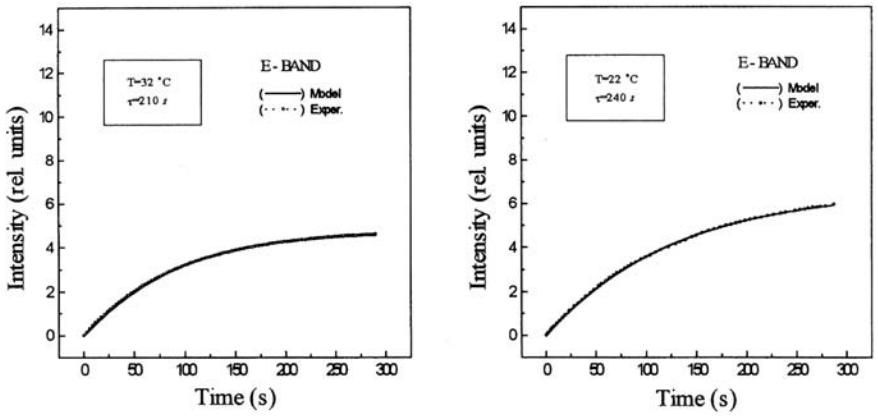


Fig. 3 — The fastest change on the delayed fluorescence induction curve (shown in **Figures 1a—2a**), a millisecond „band" *A*, recorded by the storage oscilloscope (as described in experimental part) at three different time scales: 20 *ms* (up), 500 *ms* (middle) and 5*s* (down).



k4	4,80283	err	0,00936
k5	0,01098	err	0,00006
χ^2			0,00061
Correlation			0,99982
Confidence			0,95
F=0,0011			
F_T=1,6557			
F<F_T			

k4	6,63111	err	0
k5	0,00778	err	0
χ^2			0
Correlation			1
Confidence			0,95
F=0,00014			
F_T=1,6557			
F<F_T			

Fig. 4 — Mathematical modelling of the *E* band shown in **Figure 1(a)** (left), and **Figure 2a** (right). The modelling was done according to **Eq. (5)**. The meaning of bottom signals is: k_4 (= M_0) and k_5 — pseudo-rate constants values with their errors (err); the rest is the same as for **Fig. 1b**.

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МАТЕМАТИЧКО МОДЕЛИРАЊЕ КОМПОНЕНАТА ИНДУКЦИОНЕ КРИВЕ ЗАКАСНЕЛЕ ФЛУОРЕСЦЕНЦИЈЕ ХЛОРОФИЛА

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Резиме

У раду су коришћене методе математичког моделирања индукционих компонената на које је разложена индукциона крива закаснеле флуоресценције (ЗФ) хлорофила. Индукционе компоненте су експериментално доказане и повезане су са електрохемијским градијентом (ECG) који се ствара на тилакоидној мембрани листа кукуруза.

Индукционе компоненте C и D су фитоване коришћењем модела консекутивних реакција првог реда (A → B → C) у којем оне играју улогу међупродукта (B). У том смислу индукционе компоненте можемо представити као:

$$(\text{„}A_1 \textit{ стање}\text{”})_{ECG} \xrightarrow{k_1\,(C\ компонента)} C\ компонента \xrightarrow{k_2\,(C\ компонента)} продукат$$

$$(\text{„}A_2 \textit{ стање}\text{”})_{ECG} \xrightarrow{k_1\,(D\ компонента)} D\ компонента \xrightarrow{k_2\,(D\ компонента)} продукат$$

Почетна ECG „стања” (A_1 и A_2) се разликују и зависе од дужине држања сегмента листа у мраку пре почетка интермитентног осветљавања. Постојање стационарног стања — нивоа индукционе криве ЗФ хлорофила захтева увођење Е-компоненте. Ова компонента, после дужег одигравања индукционих процеса, достиже ниво сатурације. У том смислу компонента Е може бити представљена моделом једне (или двеју паралелних) реакције првог реда):

$$(\text{„}A_3 \textit{ стање}\text{”})_{ECG} \xrightarrow{k_1\,(E\ компонента)} E$$

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SOME PHOTOSYNTHETIC PARAMETERS OF ALFALFA (*Medicago sativa* L.) LEAVES AT DIFFERENT PHENOLOGICAL STAGES AND IN DIFFERENT CUTTINGS

ABSTRACT: Five alfalfa genotypes of different origin, height, resistance, and yield level were used in the study. The plants were grown under field conditions and the apical leaves were sampled for physiological analysis at three phenological stages: 1. stage of intensive vegetative growth; 2. emergence of flower buds; and 3. flowering. The three stages were monitored during the second (May-June), third (June-July), and fourth (August) cutting. The maximum rate of net photosynthetic oxygen evolution depended on the cutting as well as the phenological stage. Genotypic divergence was less pronounced. Photosynthetic rates were lowest at flowering and highest at the stage of intensive vegetative growth. In all the genotypes the highest rate of dark respiration was recorded at the stage of intensive growth in the second cutting. Distribution of pigments in the various phenological phases varied according to the cutting. In the second cutting, there was a significant increase in the concentration of photosynthetic pigments between the stage of intensive growth and flowering. The amount of accumulated organic matter was significantly higher in the fourth cutting than in the second and third. The genotypes did not exhibit large divergence regarding the average leaf organic matter content.

KEY WORDS: *Medicago sativa* L., cuttings, phenological stages, rate of photosynthesis, pigment concentrations, dry matter.

INTRODUCTION

The cultivated or blue alfalfa (*Medicago sativa* L.) is one of the oldest and most widely grown perennial forage crops. It is grown successfully in both regions with limited soil moisture and those with high or low temperature as well as in regions where the soil is saline [Safarnejad et al., 1996]. Tolerance to stress conditions and high organic production (high yields and protein quality in particular) are characteristics that make this crop species an ir-

replaceable source of livestock feed. Alfalfa is a legume that leaves large amounts of nitrogen (N) in the soil after the plowing under of an alfalfa field, amounts large enough to satisfy the N requirements of crops that are grown after it, such as wheat, barley (during the winter), and various vegetables [Honeycutt et al., 1996]. Because of its ability to prevent soil erosion and, at the same time, enrich the soil with N, alfalfa is also grown in rotation with sunflower, maize, and other crop species [Kandel et al., 1997].

Alfalfa is fed to livestock as hay, silage, and, less often, green forage. The total yield of alfalfa dry matter includes a high percentage of crude protein faction (18—22% on average), while the ash content is about 10% [Hussain et al., 1995]. Commercial utilization of alfalfa begins in the second year of growing, and the best time for cutting is the beginning of flowering, when the dry matter content is highest [Đukić and Erić, 1995].

Time of cutting, i.e. phenological phase, has the largest effect on dry matter yields and quality. A several-day-delay in cutting can reduce protein quality (digestibility) and protein content by as much as 20 g/kg [Buxton, 1996]. The number of cuttings per season is another significant yield-determining factor, although a larger amount of dry matter does not necessarily translate into a higher dry matter nutritive value. Selection and breeding of alfalfa are directed towards developing genotypes with improved seed and vegetative mass productivity as well as towards increasing the percentage contribution of crude proteins, which is of great nutritive importance. Since the production of organic matter results from photosynthesis, the development of genotypes with increased photosynthetic potential can be a highly significant trend in plant breeding. Hill et al. (1988) state that in many crop species yields have been increased as a result of modifying regulatory processes, as opposed to assimilative ones. Such breeding trends are aimed at increasing seed yield without augmenting the vegetative mass (a higher harvest index). In alfalfa, what matters is the vegetative yield, it is the assimilative processes that need to be altered in the process of breeding and selection instead of the regulatory ones. Therefore, identification of alfalfa genotypes that, as potential parents, would be the carriers of genes responsible for the expression of higher photosynthetic potentials is highly desirable in breeding programs. The amount of organic matter that accumulates from the various cuttings during the growing season as well as the dynamics of the removal of mineral elements at different phenological stages significantly affect the direction of breeding and they are important in defining the alfalfa ideotype.

MATERIALS AND METHODS

Five alfalfa genotypes of different origin, height, resistance, and yield level were used in the study: Banat (Yugoslavia), SIN II (Yugoslavia), SIN III (Yugoslavia), Orca (France), and Du Puits (France).

The plants were grown under field conditions, using conventional cultural practices.

The apical leaves were sampled for physiological analysis at three phenological stages: 1. stage of intensive vegetative growth; 2. emergence of flower buds; and 3. flowering. The three stages were monitored during the second (May-June), third (June-July), and fourth (August) cutting.

The maximum rate of net photosynthetic oxygen evolution at light saturation is one of the parameters that define the intensity of photosynthesis, while rate of dark respiration can be expressed *via* the rate of oxygen uptake in the dark. Both of these parameters were determined polarographically on leaf segments suspended in the buffer pH 7.6—7.8 containing 10 mM $NaHCO_3$, using the Hansatech DW1 electrode [Jones and Osmond, 1973; Walker, 1987].

The concentration of chloroplast pigments — chlorophyll a, chlorophyll b, and total carotenoids — was determined spectrophotometrically following extraction in absolute acetone and expressed as mg g^{-1} dry matter [Wettstein, 1957].

Leaf dry matter content was measured after drying at 105°C and expressed as percentage of fresh leaf mass.

The data were statistically processed using the analysis of variance for the three-factorial trial (ANOVA). The comparison of the treatments was done using the Duncan's test (multiple interval test). The testing was performed for a significance level of $p<0.05$ and the values of the parameters were ranked and marked with letters. Values with the same letter did not differ significantly. The LSD interactions (genotype (A) x cutting (B) x phenological phase (C)) for $p<0.05$ enabled comparisons for each individual value.

RESULTS AND DISCUSSIONS

Net photosynthetic rate

Carbon paths in the plant as well as carbon uptake, incorporation into organic molecules, transport, redistribution, and accumulation all begin with photosynthesis. Understandably, therefore, the determination of photosynthetic parameters is of primary importance in determining the characteristics of an ideotype, especially in crop species grown for commercial purposes. Such an ideotype would definitely have to have good photosynthetic parameters, and the development of high-yielding genotypes is based on the identification of genotypes with high photosynthetic potentials [Loomis, 1993].

The maximum rate of net photosynthetic oxygen evolution depended on the cutting as well as the phenological stage. Genotypic divergence was somewhat less pronounced (Tab. 1).

A comparison of the genotype means from the various phenological stages in the three cuttings (cutting x phenological stage) revealed that photosynthetic activity did not vary significantly in May—August (the only exception was by far the lowest level of photosynthesis at the flowering stage in the fourth cutting). Photosynthetic rates were lowest at flowering and highest, and significantly so, at the stage of intensive vegetative growth. Genotypic speci-

Tab. 1 — Rate of photosynthesis in different alfalfa genotypes in three cuttings and at three phenological stages*

Cutting	Phenolog. stage	Photosynthesis ($\mu molO_2 g^{-1} h^{-1}$)					
		genotype					
		Banat	Sin II	Sin III	Orca	Du Puits	average
II	1	785	647	578	681	639	666[a]
	2	359	478	512	614	534	499[c]
	3	357	368	471	464	557	443[d]
	average	500[c]	498[c]	520[abc]	586[a]	577[ab]	
III	1	739	624	578	647	670	651[a]
	2	573	521	562	677	562	579[b]
	3	427	458	416	427	539	453[cd]
	average	580[ab]	534[abc]	519[abc]	583[a]	590[a]	
IV	1	762	635	578	647	655	655[a]
	2	472	563	485	485	422	485[cd]
	3	336	375	422	397	414	389[e]
	average	523[abc]	524[abc]	495[c]	510[bc]	497[c]	
LSD AxBxC		107					

*1. stage of intensive vegetative growth; 2. stage of emergance of flower buds; 3. stage of flowering
Means with the same letter did not differ significantly at $p<0.05$

ficity with respect to photosynthetic activity depended on both the phenological stage and cutting. The French genotypes Orca and Du Puits had the highest rates of photosynthesis in the second and third cutting (the mean value for all three phenological phases), while the domestic genotypes Banat and SIN II did so in the fourth.

Data for maximum photosynthetic rates under light saturation in laboratory conditions showed that there was little genotypic variability; which of the genotypes had the highest value depended on the phenological phase. The conclusion that can be drawn is that it is very hard to reliably define genotypic specificity for photosynthetic activity, since the process of photosynthesis is significantly affected by factors such as the environment and the ontogenetic stage of the photosynthetic organ. The effects of these factors manifest themselves in a wide time range: the second, hour, day, month, or even the entire season. It is therefore very difficult to standardize the status of the photosynthetic organ under study regarding its age, time of measurement, and some abiotic factors so that the possible differences could be ascribed exclusively to the genotype [Austin, 1993]. In the present study, Orca can be singled out as the genotype with the highest photosynthetic potential. Parameters of photosynthesis, however, must always be considered in relation to the total leaf area. The leaf is the main photosynthetic organ, so it can be assumed that genotypes with larger total leaf area will have a greater photosynthetic capacity and hence a higher organic matter production than those with smaller area.

Small genetic variability of the photosynthetic capacity of breeding materials and limited possibilities for the utilization of this variability have had a limiting effect and have slowed down the selection for the improvement of photosynthetic traits [Simón, 1994]. For this reason, selection for better photosynthetic traits is often connected with analysis of morphological and other characteristics.

Rate of dark respiration

When conducting respiration-related genetic manipulations, it is important to make an estimate of unnecessary energy losses within the energy that supplies the vital functions in order to increase the efficiency of respiration, which is the main objective of defining genotypes with desirable traits [Penning de Vries, 1975].

In all the genotypes in our study, the highest rate of respiration was recorded at the stage of intensive growth in the second cutting (Tab. 2). The other values of respiration rate did not depend on the cutting. As plant growth and development within each cutting progressed, we noticed a tendency towards a drop in the rate of respiration, so that lowest values were recorded at flowering. The decrease of respiration rate that accompanies the ageing of alfalfa leaves comes as a result of a drop in the rate of photosynthesis at later stages of growth and a reduction of the amount of assimilates that are used as

Tab. 2 — Rate of dark respiration in different alfalfa genotypes in three cuttings and at three phenological stages*

Cutting	Phenolog. stage	Dark respiration (—μmolO_2g^{-1}h^{-1})					
		genotype					
		Banat	Sin II	Sin III	Orca	Du Puits	average
II	1	354	381	338	335	293	340^{a}
	2	216	216	227	262	250	234^{c}
	3	227	273	262	227	239	246^{c}
	average	266^{abc}	290^{a}	276^{ab}	274^{ab}	261^{abc}	
III	1	277	231	231	254	266	252^{c}
	2	219	229	239	239	271	239^{c}
	3	177	208	177	177	177	183^{d}
	average	224de	223de	216^{e}	223de	238^{cde}	
IV	1	316	306	285	295	279	296^{b}
	2	219	250	250	271	234	245^{c}
	3	172	203	172	164	166	175^{d}
	average	236^{cde}	253^{bcd}	236^{cde}	243^{bcde}	226de	
LSD AxBxC		50					

*1. stage of intensive vegetative growth; 2. stage of emergance of flower buds; 3. stage of flowering
Means with the same letter did not differ significantly at $p<0.05$

a substrate during respiration [Heichel. et al., 1988]. Genotypic divergence was less pronounced than with photosynthetic rates. Abiotic factors — most of all temperature, concentration of atmospheric CO_2, and the amount of soil nitrogen — also have a significant effect on respiration and cannot be ignored when determining genotypic specificity for this trait [Azcón-Bieto et al., 1994].

On the whole, we found significantly higher photosynthetic and metabolic rates at the stage of intensive growth.

Concentration of photosynthetic pigments — total chlorophylls and carotenoids

The photosynthetic properties of a genotype are significantly affected by the structure and function of the photosynthetic apparatus itself, i.e., by the density of the reaction centers and the photosystems in the thylakoid membranes. Not surprisingly, therefore, the concentration of photosynthetic pigments (chlorophyll a, chlorophyll b, and total carotenoids) has significant influence on photosynthesis, i.e., organic matter production. The concentration of pigments, especially carotenoids, has a significant effect on the nutritive value as well.

The results of our study showed that the distribution of pigments in the various phenological phases varied according to the cutting. In the second cutting, thus, there was a significant increase in the concentration of photosynthetic pigments between the stage of intensive growth and flowering (Tab. 3). The increase (per gram of dry matter) was particularly large just before and during flowering.

Tab. 3 — Concentration of chloroplast pigments in different alfalfa genotypes in three cuttings and at three phenological stages*

Cutting	Phenolog stage	**Chl a+b (mg g^{-1} dry matter)**					
		genotype					
		Banat	Sin II	Sin III	Orca	Du Puits	average
I	1	6.29	7.65	6.81	7.40	6.78	6.98^{de}
	2	7.78	7.18	9.64	7.89	6.39	7.77^{cd}
	3	14.19	16.98	16.53	15.37	5.14	15.64^{a}
	average	9.42^{bcd}	10.60^{ab}	10.99^{a}	10.22^{abc}	9.44^{bcd}	
II	1	6.07	5.31	8.22	6.70	7.23	6.70^{e}
	2	9.72	11.59	11.44	12.71	11.63	11.41^{b}
	3	7.62	9.48	7.86	9.21	9.42	8.42^{c}
	average	7.80^{ef}	8.79^{de}	9.17^{cd}	9.43^{bcd}	9.42^{bcd}	
III	1	6.18	6.48	7.52	7.05	7.00	6.85^{de}
	2	7.82	8.40	7.75	9.93	8.27	8.43^{c}
	3	5.66	4.29	5.67	4.84	5.32	5.15^{f}
	average	6.55^{fg}	6.39^{g}	6.98^{fg}	7.27^{fg}	6.86^{fg}	
LSD AxBxC		2.03					

Cutting	Phenolog. stage	Carotenoids (mg g^{-1} dry matter)					
		Banat	Sin II	Sin III	Orca	Du Puits	average
I	1	4.02	4.73	4.32	4.66	4.22	4.39^{d}
	2	5.09	4.79	6.06	5.42	5.90	5.45^{c}
	3	9.70	9.82	9.97	9.34	9.50	9.66^{a}
	average	6.27^{ab}	6.45^{a}	6.78^{a}	6.47^{a}	6.54^{a}	
II	1	1.19	0.93	3.88	4.19	4.58	2.95^{f}
	2	6.01	7.43	7.22	7.98	7.29	7.18^{b}
	3	4.72	5.94	5.02	5.48	5.62	5.35^{c}
	average	3.97^{dc}	4.77^{cd}	5.37^{bc}	5.88^{ab}	5.83^{ab}	
III	1	2.60	2.84	4.10	4.42	4.40	3.67^{e}
	2	4.69	5.42	4.79	6.30	5.23	5.28^{c}
	3	3.75	2.65	3.66	3.01	3.38	3.29^{ef}
	average	3.68^{e}	3.63^{e}	4.18^{de}	4.57^{cde}	4.33^{de}	
LSD AxBxC		1.51					

*1. stage of intensive vegetative growth; 2. stage of emergance of flower buds; 3. stage of flowering
Means with the same letter did not differ significantly at $p<0.05$

A comparison between data for photosynthetic activity and data for the concentration of photosynthetic pigments in the second cutting suggests a complete lack of correlation, since the highest photosynthetic activity was recorded at the stage of intensive vegetative growth, when the concentration of pigments was at its lowest. The lack of correlation between photosynthetic parameters and pigment concentration has been noticed previously in other crop species [Edwards et al., 1993; Pajević, 1997]. Therefore, the absence of the expected significant positive correlation between photosynthetic activity and the concentration of photosynthetic pigments can be attributed to a balanced absorption of photons on the part of antenna pigments and pigments from the reactive centers.

The average concentration of photosynthetic pigments at the three stages of growth and development revealed a low level of genotypic variability.

Leaf dry matter content

The amount of accumulated organic matter was significantly higher in the fourth cutting than in the second and third (Tab. 4).

The genotypes did not exhibit large divergence regarding the average leaf organic matter content. Noticeably, the genotype with the highest dry matter differed from cutting to cutting. Still, SIN II and Orca may be singled out as genotypes with an increased potential for the accumulation of organic matter. However, although the rate of organic matter accumulation in leaves varied from one growth stage to another, in all of the genotypes the maximum was always recorded at flowering. At the flower bud stage in the third cutting, there was a certain drop in organic matter synthesis in all the genotypes, which

Tab. 4 — Leaf dry matter content in different alfalfa genotypes in three cuttings and at three phenological stages*

Cutting	Phenolog. stage	Dry matter (%)					
		genotype					
		Banat	Sin II	Sin III	Orca	Du Puits	average
I	1	19.5	20.5	20.5	21.0	21.5	20.6^{e}
	2	23.0	24.5	21.0	23.0	21.5	22.6^{cd}
	3	23.0	24.0	23.0	25.0	21.5	23.3^{c}
	average	21.83^{de}	23.00^{d}	21.50^{de}	23.00^{d}	21.50^{de}	
II	1	21.0	19.5	20.5	22.5	22.0	21.1^{de}
	2	21.0	17.5	18.5	15.5	18.0	18.1^{f}
	3	25.0	24.5	24.0	23.0	22.5	23.8^{c}
	average	22.33^{de}	20.50^{e}	21.00^{de}	20.33^{e}	20.83^{de}	
III	1	20.5	20.0	20.5	22.0	22.0	21.0^{de}
	2	28.0	30.0	26.5	26.0	28.0	27.7^{b}
	3	38.5	48.0	34.0	39.5	39.0	39.8^{a}
	average	29.00^{bc}	32.67^{a}	27.00^{c}	29.17^{b}	29.67^{b}	
LSD AxBxC		3.49					

*1. stage of intensive vegetative growth; 2. stage of emergance of flower buds; 3. stage of flowering
Means with the same letter did not differ significantly at $p<0.05$

was most probably, caused by the action of environmental factors, such as high temperatures and water deficiency.

Knowing the dynamics of organic matter accumulation is of great importance in determining the optimum cutting interval within a season. The re-growth of the above-ground plant parts after cutting is a complex process in which the final yield of organic matter is determined by interactions between environmental and endogenous plant factors (amount of reserve substances in the root, remaining number of active meristem tips, etc.) [Avice et al., 1997]. Cutting frequency is therefore critical in determining the production of dry matter. Thus, Borowiecki et al. (1996) report that the optimum interval for dry matter accumulation between two cuttings is 45 days — more frequent cuttings reduce yields considerably. According to Đukić (1997), either the bud stage or flowering is the optimum time for cutting in terms of dry matter accumulation in the above-ground plant parts. The results of our experiment support that conclusion.

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НЕКИ ФОТОСИНТЕТИЧКИ ПАРАМЕТРИ ЛИСТОВА ЛУЦЕРКЕ (*Medicago sativa* L.) У РАЗЛИЧИТИМ ФЕНОЛОШКИМ ФАЗАМА И ОТКОСИМА

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Резиме

Пет генотипова луцерке (*Medicago sativa* L.) различитог географског порекла, морфолошких карактеристика и приноса коришћено је за изучавање фотосинтетичких карактеристика и динамике накупљања органске материје у листовима. Параметри су одређивани током три пораста (откоса) у три фенолошке фазе: 1. фаза интензивног вегетавионог пораста; 2. фаза појаве цветних пупољака; 3. фаза цветања.

Генотипска специфичност у погледу фотосинтетичке активности зависила је од испитиване фенолошке фазе и пораста и била је слабо изражена. У фази интензивног вегетативног пораста забележене су значајно највише фотосинтетичке брзине, док су у фази цветања забележене најниже. Брзина респираторног усвајања кисеоника такође је била највиша у фази интензивног вегетативног пораста. Није била изражена генотипска дивергентност у односу на просечну концентрацију фотосинтетичких пигмената. У другом откосу постојао је значајан пораст концентрације фотосинтетичких пигмената од фазе интензивног вегетативног пораста до фазе цветања. Није било корелације између фотосинтезе и концентрације пигмената.

Количина акумулиране органске материје била је значајно највећа у четвртом порасту у односу на други и трећи.

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NITROGENASE ACTIVITY IN PURE CULTURE OF *AZOTOBACTER CHROOCOCCUM* AND IN ASSOCIATION WITH SUGAR BEET

ABSTRACT: The objective of this experiment was to determine nitrogenase activity in several strains of *Azotobacter chroococcum* and in sugar beet plants inoculated with these strains. Used in the study were five *Azotobacter chroococcum* strains (2, 3, 6, 8, and 14) isolated from the rhizosphere of sugar beets grown at the experiment field of the Institute of Field and Vegetable Crops in Novi Sad. Nitrogenase activity in a pure culture of *Azotobacter chroococcum* as well as in an association of sugar beet with these bacteria was determined according to Hardy et al. (1968). In both cases, the highest nitrogenase activity was recorded in treatments with strain 14.

KEY WORDS: *Azotobacter chroococcum*, association, nitrogenase activity, strain, sugar beet.

INTRODUCTION

The genus *Azotobacter* consists of free-living nitrogen-fixing rhizosphere bacteria that belong to a group of bacteria termed plant growth-promoting rhizobacteria (PGPR) because of the beneficial effects they exert on plant growth. Due to the the plant growth-promoting properties of this genus, numerous studies on *Azotobacter* ecology, physiology, and biochemistry have been carried out.

The process of nitrogen fixation in these aerobic soil bacteria has been studied for many years, but it was only in 1980 that it was established that these diazotrophs contain three genetically different nitrogenases [Bishop and Premakumar, 1980]. One of these enzymes is the well characterized, conventional nitrogenase 1, which contains Mo.

The activity of this nitrogenase (EC. 1.18.2.1) is determined by the acetylene-reduction method, which is very sensitive when measuring nitrogenase activity at a given moment but offers limited possibilities when determining

the effects of N_2-fixation integrated over time. Nitrogenase activity in pure cultures of *Azotobacter chroococcum* strains has been studied rather extensively (Hong et al., 1986; Abdalla et al., 1992; Dilwort et al., 1993; Garciabarrinuevo et al., 1993).

The effects of inoculation with *Azotobacter chroococcum* strains on nitrogenase effectiveness in maize and wheat were studied by Hegazi et al. (1986) and Gašić et al. (1990), respectively, while Mrkovački et al. (1995, 1997) studied these effects in inoculated sugar beet plants grown *in vitro*.

The objective of this experiment was to determine nitrogenase activity in several strains of *Azotobacter chroococcum* and in sugar beet plants inoculated with these strains.

MATERIAL AND METHODS

Used in the study were five *Azotobacter chroococcum* strains (2, 3, 6, 8, and 14) isolated from the rhizosphere of sugar beets grown at the experiment field of the Institute of Field and Vegetable Crops in Novi Sad. The strains were grown on the Fedorov medium with sucrose.

Determination of nitrogenase in pure Azotobacter culture

Nitrogenase activity in pure culture of *Azotobacter chroococcum* was determined according to Hardy et al. (1968) by preparing an *Azotobacter chroococcum* inoculum (pre-culture) in a liquid Fedorov medium for 20 hours at 30°C with gentle shaking. Under sterile conditions, 2 ml of this pre-culture were transferred into sterile penicillin flasks, which were then closed with rubber stoppers. Following this, the air was pumped out of the flasks using a gas syringe, after which acetylene (10% of the test tube volume) was added to them. The flasks were then placed on a shaker (30°C). After 24 hours, a 0.1 ml gas sample was taken and analyzed on a gas chromatographer (Hewlett Packard 5480A). Finally, the areas of the ethylene peaks were determined.

Determination of nitrogenase in plants inoculated with Azotobacter

The seed of sugar beet (Hy-11) was inoculated with 0.5 ml of liquid *Azotobacter chroococcum*. culture (10^9 cells/ml) and placed in test tubes containing a sterile agarized nitrogen-free medium (Murashige, Skoog, 1962). The experiment had three replications. After four weeks of *in vitro* plant growth, the air was taken out of the test tubes and replaced with acetylene (10% of the test tube volume) to determine nitrogenase activity. The samples were then incubated for 24 hours at 30°C, after which a 0.1 ml gas sample was taken from each test tube and analyzed on a gas chromatographer

(Hewlett Packard 5480A). This was followed by the determination of the areas of the ethylene peaks.

RESULTS AND DISCUSSION

A number of studies have reported differences in nitrogenase activity between different *Azotobacter* strains (Shawky, 1982; Nikogosyan, 1984; Hong et al., 1986). In the present study (Fig. 1), the various *Azotobacter* strains differed greatly with regard to their nitrogenase activity. The strains could be recognized by the level of their nitrogenase activity. The highest nitrogenase activity was recorded in strain 14, the lowest in strain 6.

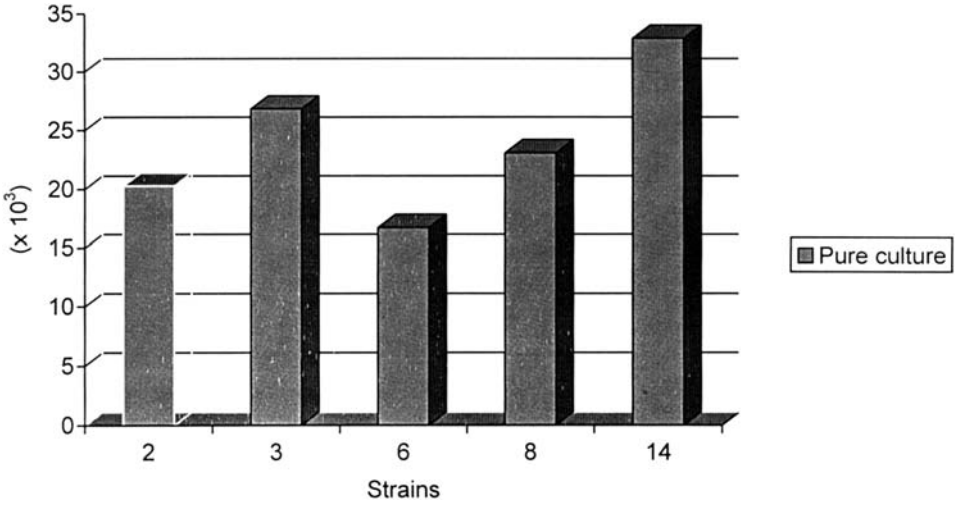

Fig. 1 — Area of ethylene peaks in pure cultures of *Azotobacter chroococcum*

We were able to determine nitrogenase activity in all the inoculated plants (Fig. 2). As for the inoculated plants, the highest nitrogenase activity, i.e., the largest area of ethylene peaks, was found in plants inoculated with strain 14.

The results of Gašić et al. (1990) suggest that the effect of *Azotobacter* strains on nitrogenase activity is smaller when they are associated with plants (wheat) than in pure culture, where the differences among the strains are greater (Hong et al., 1990).

The results of the present study, on the other hand, indicate that nitrogenase activity has the same tendency in the pure cultures of *Azotobacter chroococcum* as in association of this bacterium with sugar beet. Strain 14 had the highest nitrogenase activity in both the inoculated plants and pure cultures.

However, it must be born in mind that in the development of a plant-*Azotobacter* association a major role is played not only by the microorganism but by the plant genotype as well. Therefore, it remains to be seen in further studies how strain 14 will behave in association with other sugar beet hybrids.

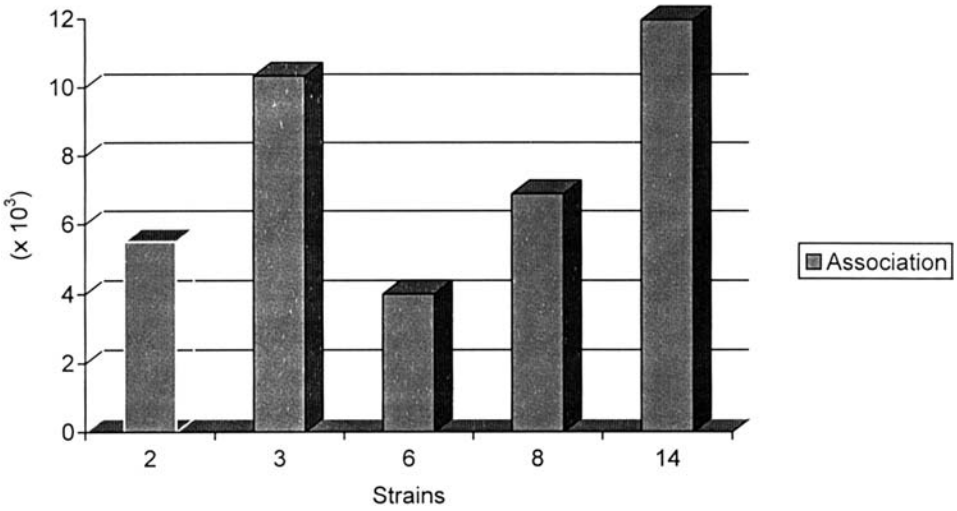

Fig. 2 — Area of ethylene peaks in association of *Azotobacter chroococcum* strains and sugar beet plants

Combined nitrogen inhibits *Azotobacter*'s nitrogenase activity and increases the concentration of nitrogen in bacterial cells. In the present paper, we obtained nitrogenase activity without adding any N to the medium. In Mrkovački et al. (1997), the studied associations of sugar beet plants and *Azotobacter* strains were characterized by significant differences in plant growth, nitrogenase activity, and N_2 fixation. These differences improved the selection of active strains.

CONCLUSION

The studied *Azotobacter* strains differed with regard to nitrogenase activity in both the pure cultures and in the association with sugar beet.

Strain 14 had the highest nitrogenase activity in both the inoculated plants and pure culture.

The differences in nitrogenase activity enable the selection of highly effective strains.

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АКТИВНОСТ НИТРОГЕНАЗЕ У ЧИСТОЈ КУЛТУРИ *AZOTOBACTER CHROOCOCCUM* И У АСОЦИЈАЦИЈИ СА ШЕЋЕРНОМ РЕПОМ

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Резиме

Процес азотофиксације аеробних земљишних бактерија *Azotobacter* проучаван је много година, али је тек 1980. установљено да овај диазотроф поседује 3 генетски различите нитрогеназе. У раду је коришћено 5 сојева (2, 3, 6, 8 и 14) *Azotobacter chroococcum* изолованих из ризосфере шећерне репе гајене на огледним пољима Института за ратарство и повртарство у Новом Саду. Сојеви су изоловани и гајени на подлози Фјодора са сахарозом. Нитрогеназа у чистој култури *Azotobacter chroococcum* одређивана је методом H a r d y et al. Семе шећерне репе (Ну-11) инокулисано је са 0.5 ml течне културе *Azotobacter chroococcum* густине 10^9 ћелија/ml. Добијени резултати у овом раду показали су да постоји иста тенденција активности нитрогеназе у чистим културама *Azotobacter chroococcum* и у асоцијацији са биљком шећерне репе. Сој *Azotobacter chroococcum* 14 који је показао највећу нитрогеназну активност у асоцијацији са биљком остварио је такође највећу активност нитрогеназе у чистој култури. Разлике у активности нитрогеназе омогућавају селекцију високо ефективних сојева.

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NUMBER OF MICROORGANISMS IN THE SOIL UNDER DIFFERENT BEAN GENOTYPES

ABSTRACT: Numbers of various microorganisms (total number of microorganisms, numbers of ammonifiers, azotobacters, fungi, actinomycetes and other free nitrogen-fixing bacteria) have been followed in a black meadow soil planted to eight bean varieties (Panonski gradištanac, Vukovarski težak, Zlatko, Belko, Dvadesetica, Sremac, Royal Duch and Slavonski žuto zeleni). Immediately before planting, been seeds have been inoculated with a microbiological preparation (NS-Nitragin for beans). Numbers of the studied microorganisms have decreased in the course of vegetation in relation to the situation immediately before planting, with the exception of the total number of bacteria which, at the end of the vegetation, reached the original situation immediately before the planting. On average for all varieties, the numbers of azotobacters and ammonifiers were lowest at the stage of flowering. The obtained results have shown that root exudates and plowed under corn stalks affected the numbers of the microorganisms under study.

KEY WORDS: number of microorganisms, azotobacters, free nitrogen-fixing bacteria, ammonifiers, fungi, actinomycetes, bean genotype, black meadow soil.

INTRODUCTION

Soil is a natural environment for a number of microorganisms — bacteria, algae, fungi, actinomycetes. These microorganisms take part in the biochemical, biological and chemical processes in soil.

Each soil type has a specific microflora which changes in dependence of geographic and ecological factors, method of soil tillage and mineral fertilization, crop, herbicides applied, etc. (Mišustin, 1966; Sarić et al., 1983; Mrkovački, 1985; Milić et al., 1997; Milić, 1999). There is a relationship between pedogenetic and microbiological properties of soil; taken together, they provide the potential and effective capacity for plant growing.

Numerous authors have described properties of black meadow soils (Živković and Ristić, 1963; Živković, 1965; Živković et al., 1967,

1968); microorganisms inhabiting profiles of the black meadow soils were discussed by Sarić (1972).

The objective of this paper was to assess the numbers of various microorganisms in a black meadow soil planted to bean genotypes.

MATERIAL AND METHOD

This study covers the assessment of the numbers of certain groups of microorganisms in the black meadow soil in the location of Nova Gajdobra. Experiments were established in a plot planted to corn the previous year. Soil tillage was conventional, without mineral fertilization. Eight bean genotypes (Belko, Zlatko, Sremac, Dvadesetica (developed at the Institute of Field and Vegetable Crops, Novi Sad), Slavonski žuto zeleni and Vukovarski težak (Agricultural Station Vukovar), Panonski gradištanac (Center for Vegetables, Smederevska Palanka) and Royal Dutch (from the Netherlands) were planted in a plot of 0.35 ha (650 x 5.6 m).

Soil samples for microbiological analyses were taken from the soil layer 0—20 cm, before planting and at the stages of flowering and full maturity, the samples for agrochemical analyses before planting and the end of the season.

All seeds were inoculated with a microbiological fertilizer NS-Nitragin that contains *Rhizobium leguminosarum bv. phaseoli* strains specific for the bean. The preparation was produced at the Institute of Field and Vegetable Crops in Novi Sad.

Microbiological status of the soil was analyzed by the method of Pochon and Tardieux (1962), for the total number of microorganisms in soil agar, the number of ammonifiers on the mesopeptonic agar, the number of azotobacters and free nitrogen-fixing bacteria on Fedorov's substrate, the number of fungi on Chapek's agar and the number of actinomycetes on Krasiljnikov's synthetic agar. The numbers of the microorganisms were calculated per gram of absolutely dry soil, using the number of microorganisms in the soil immediately before planting (Ø) as the standard.

RESULTS AND DISCUSSION

Regarding their areage in the Vojvodina Province, the black meadow soils take the second position, after chernozems. According to Manojlović (1988), agrochemical analyses performed before planting indicated that the studied soil type was slightly calcareous, at the lower limit of the medium provision with humus, neutral, with a high potassium content and optimum phosphorus content. At the stage of full maturity of the analyzed bean genotypes, the soil was alkaline and with somewhat increased contents of potassium and phosphorus in relation to the original situation (Table 1).

The data presented in Table 2 show that the numbers of the microorganisms in the soil layer 0—20 cm decreased progressively in relation to the situation before the planting. The total number of bacteria in the soil before plant-

Tab. 1 — Agrochemical analyses of soil

Variant	$CaC0_3$ %	Humus %	N %	KCl pH	H_2O pH	mg/100 g soil	
						P_2O_5	K_2O
Before planting Ø	1,62	2,65	0,132	6,99	7,96	18,30	30,0
End of vegetation	3,08	3,45	0,172	7,47	8,21	23,79	39,0

ing was 746 x 10^7 per 1 gram of absolutely dry soil. At the stage of flowering, the numbers went down. On average for all genotypes, the largest numbers were recorded for the total number of bacteria and the number of free nitrogen-fixing bacteria, 267.5 x 10^7 and 302.87 x 10^6 per 1 g of soil, respectively. The numbers of the azotobacters and ammoinifiers were 50.87 x 10^2 and 81 x 10^7 per 1 g of soil, respectively. The numbers of fungi and actinomycetes were somewhat lower than in the control variant (Ø).

The lowest total number of bacteria was obtained with the variety Slavonski žuto zeleni (144 x 10^7 per 1 g of soil), the highest with Vukovarski težak (446 x 10^7 per 1 g of soil) (Table 2).

Tab. 2 — The number of microorganisms in the soil at the stage of flowering of bean plants

Variant	Total number of bacteria x 10^7	Fungi x 10^4	Actinomycetes x 10^4	Ammonifiers x 10^7	Free nitrogen-fixing bacteria x 10^6	Azotobacter x 10^2
Before planting Ø	746	11.38	10.57	332	641	207
Panonski gradištanac	252	3.14	12.58	60	293	82
Vukovarski težak	446	6.96	6.96	90	380	29
Zlatko	315	11.41	760	126	350	120
Belko	237	10.03	9.01	91	253	49
Dvadesetica	220	7.95	11.57	38	274	35
Sremac	269	4.65	11.61	128	392	29
Royal Duch	257	13.71	10.24	71	299	28
Slavonski žuto zeleni	144	9.84	5.8	44	182	35
Average	267.50	9.26	9.37	81	302.87	50,87

On average for the bean genotypes under study, the largest total number of bacteria (74.1 x 10^7 per 1 g of soil) and the lowest number of fungi (6,33 x 10^4 per 1 g of soil) were obtained at the end of vegetation of bean plants. The number of actinomycetes could not be identified with the dilutions used although the inoculation of soybean had decreased the number of actinomycetes (Milić et al., 1997; Milić, 1999). The lowest total numbers of microorga-

nisms were registered with the varieties Panonski gradištanac and Vukovarski težak, 521 x 10^7 and 551 x 10^7 per 1 g of soil, respectively, the highest with Royal Dutch and Slavonski žuto zeleni, 1732 x 10^7 and 922 x 10^7 per 1 g of soil, respectively (Table 3).

Tab. 3 — The number of microorganisms in the soil at the end of vegetation of bean plants

Variant	Total number of bacteria x 10^7	Fungi x 10^4	Actinomycetes x 10^4	Ammonifiers x 10^7	Free nitrogen-fixing bacteria x 10^6	Azotobacter x 10^2
Before planting Ø	746	11.38	10.57	332	641	207
Panonski gradištanac	521	15.72	0.00	58	111	65
Vukovarski težak	551	6.70	0.0	132	193	131
Zlatko	944	5.73	0.00	127	198	120
Belko	696	2.25	0.00	74	279	68
Dvadesetica	694	6.64	0.00	96	138	55
Sremac	790	4.54	0.00	97	173	102
Royal Duch	1732	4,52	0,00	298	147	96
Slavonski žuto zeleni	922	4.57	0.00	160	253	34
Average	741	6.33	0.00	130.25	186.50	83,87

The obtained results show that the values of the total number of bacteria, free nitrogen-fixing bacteria and ammonifiers were high, as well as that the root exudates of the bean genotypes had an effect on the numbers of the microorganisms.

According to Jarak et al. (1994), inoculation of string beans increases the number of azotobacters in the rhizosphere. Sarić et al. (1983) reported that corn harvest residues inhibit the development of azotobacters. On average for the tested bean varieties, the number of azotobacters was lowest at the stage of flowering. The numbers of the free nitrogen-fixing bacteria and ammonifiers were also reduced. At the end of the vegetation, the total number of bacteria was at the level of that established before the planting of the bean varieties.

All bean genotypes in the experiment tended to reduce the numbers of microorganisms under study. The reductions in the number of microorganisms observed in the course of bean vegetation may have been caused by toxic substances developing in the course of decomposition of corn harvest residues. As the plowed under corn residues decompose slowly, the release of organic matter and nitrogen is delayed, resulting in a reduction of the biological activity of the soil.

CONCLUSIONS

Following conclusions were drawn on the basis of the results obtained.

— The dynamics of increase/decrease of the studied microorganisms depended on the bean genotype.

— In the course of the vegetation, the numbers of the microorganisms under study decreased in relation to the situation before the planting.

— On average for all bean genotypes, the numbers of azotobacters and ammonifiers were lowest at the stage of flowering.

— The total number of bacteria at the end of the vegetation was similar to that established immediately before the planting.

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БРОЈ МИКРООРГАНИЗАМА У ЗЕМЉИШТУ КОД РАЗЛИЧИТИХ ГЕНОТИПОВА ПАСУЉА

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Резиме

У раду је испитивана заступљеност појединих група микроорганизама (укупан број бактерија, број амонификатора, азотобактера, гљива, актиномицета и осталих слободних азотофиксатора) у земљишту типа ливадска црница у току вегетације осам генотипова пасуља (Панонски градиштанац, Вуковарски тежак, Златко, Белко, Двадесетица, Сремац, Royal Duch и Славонско жуто зелени). Непосредно пред сетву семе је инокулисано микробиолошким препаратом НС-Нитрагином за пасуљ. Оглед је постављен на локалитету Нова Гајдобра у току 1998. године. На почетку непосредно пред сетву и у фази зрелости пасуља урађене су агрохемијске анализе земљишта у слоју од 0—20 cm (Таб. 1). Микробиолошке анализе земљишта урађене су непосредно пред сетву, у фази цветања пасуља и на крају вегетације (Таб. 2. и 3). Добијени резултати показују да коренске излучевине испитиваних генотипова пасуља као и заорана кукурузовина утичу на заступљеност испитиваних група микроорганизама. Број испитиваних група микроорганизама у току вегетације биљака опада у односу на почетно стање пре сетве. У фази цветања, у просеку за све сорте пасуља број азотобактера и амонификатора је најмањи у односу на њихов број у испитиваним роковима, док је број укупних бактерија на крају вегетације достигао број на нивоу почетног броја.

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EFFECT OF LEAD AND CADMIUM ON CALLUS GROWTH AND DRY MATTER CONTENT IN ZYGOTIC EMBRYO CULTURE OF WHEAT (*Triticum aestivum* L.)

ABSTRACT: The objective of this study was to determine the effects of lead and cadmium on callus growth and callus tissue dry matter content. Five concentrations of Pb and Cd were studied: 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M nutrient medium. Calluses obtained from mature zygotic wheat embryos (*Triticum aestivum* L., *cv*, Balkan) were used in the experiment. Isolated embryos were grown on a modified MS [Murashige and Skoog, 1962] nutrient medium. Callus tissue growth was observed during cultivation, and then, 30 days after isolation, callus fresh weight and dry matter content were measured. The measurements showed that there were significant differences between lead and cadmium regarding their effects on callus induction, callus growth, and callus tissue dry matter content. When lead and cadmium were applied at the highest concentration (10^{-3} M), callus fresh weight decreased significantly (by 42% with lead and 87% with cadmium) relative to the control, which was not treated with heavy metals. However, the same concentration increased callus tissue dry matter content by 9.2% (lead) and 9.8% (cadmium). In relation to the control, this was an increase by 22.7% and 30.7%, respectively. The effects of the other, lower concentrations, were considerably less pronounced, although the 10^{-6} M concentration of lead and cadmium did have a stimulative effect on callus tissue growth.

KEY WORDS: cadmium, embryo culture, lead, wheat.

INTRODUCTION

Cadmium and lead belong to the group of non-essential heavy metals which, at higher concentrations, have highly toxic effects on plants. The phytoxicity of heavy metals results from their influence on various metabolic and chemical processes in plants [Van Assche et al., 1988; Kastori et al., 1997]. Studies have shown that lead and cadmium directly or indirectly inhibit such physiological processes as photosynthesis, respiration, water regimen, nitrogen metabolism, etc. [Van Assche and Clijsters, 1990; Hernandez et al., 1997; Lagriffoul et al., 1998].

Different plant species respond differently to the presence of high heavy metal concentrations [John, 1973]. Tomato, for instance, is relatively tolerant to excess cadmium [Bingham et al., 1975] while wheat and soybean are able to withstand the presence of lead in the nutrient substrate [Diehl et al., 1983; Kastori et al., 1991]. At the same time, a number of authors have reported that certain genotypes of the same species react differently to the presence of heavy metals in the soil [Florijn and Van Beusichem, 1993; Hinsley et al., 1978; Foy, 1995]. These differences among genotypes make it possible for breeders to develop varieties and hybrids with higher tolerance to metals and, even more importantly, lower accumulation of heavy metals, thereby reducing their introduction into the food chain [Kastori et al., 1997].

Lately, use of *in vitro* methods for identifying genotypes tolerant to heavy metals has been on the increase, since studies have shown that tolerance determined at the level of the whole plants is also valid at the level of the callus, i.e., that it is manifested at the cell level as well. Such studies have been carried out in *Licopersicum esculentum* [Meredith, 1978], *Sorghum bicolor* [Smith et al., 1983], *Medicaeo sativa* [Parrot and Bouton, 1990], *Triticale* and *Triticum aestivum* [Karsai et al., 1994].

The present study investigated the effects of lead and cadmium on callus growth in wheat as well as on the dry matter content of this crop's calluses, in order to assess the possibility of using *in vitro* embryo cultures to identify wheat genotypes tolerant to these metals.

MATERIAL AND METHODS

The high-yielding winter wheat (*Triticum aestivum* L.) variety Balkan was used for the isolation of mature embryos.

Air-dry mature wheat grains were immersed in distilled water for four hours, after which the materials were sterilized using a procedure described in our previous papers [Šesek and Kondić, 1997; Kondić et al., 1998]. Isolated embryos were inoculated onto a modified MS [Murashige and Skoog, 1962] nutrient medium to which Cd and Pb were added in five different concentrations (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M). The control group of embryos was inoculated onto an MS medium that contained no heavy metals.

During the one month of cultivation on these mediums, callus survival and regenerant survival were observed on a ten-day basis. At the end of the period, fresh callus weight and dry matter content were determined.

The results were statistically processed by the analysis of variance and the significance of differences between particular treatments was determined using the LSD test.

RESULTS AND DISCUSSION

The results of the study have shown that there are significant differences between lead and cadmium with regard to their effects on the induction and

growth of callus tissue as well as callus dry matter content. Cadmium was found to have had a greater inhibitory effect on callus induction and growth in wheat than lead. The differences were greatest with the highest concentration of these two metals (10^{-3} M). In the case of Cd, this concentration had a lethal effect on the isolated embryos.

With 10^{-3} M Cd, a very small number of calluses was formed (30.9%), and their fresh weight was reduced by 87% relative to the fresh weight of the calluses from the control treatment. These calluses differed from those in the control treatment in terms of their appearance too — they were compact and dehydrated. With 10^{-3} M Pb, 88.2% of the isolated embryos formed calluses that looked normal and had the fresh weight 42% lower than that in the control treatment.

Concerning the influence of the two metals on fresh callus weight (Tab. 1), it was observed that the 10^{-6} M concentration had a stimulative effect on callus growth, as 10^{-6} M Pb and 10^{-6} M Cd increased fresh callus weight by 25 and 9%, respectively, relative to the control. Any further increase in Pb and Cd concentrations, however, had an inhibitory influence on callus growth, so that 10^{-3} M Pb produced the fresh callus weight of 61.3 mg and 10^{-3} Cd the fresh callus weight of 14 mg. Another difference between lead and cadmium was that Pb had an inhibitory effect only at the highest concentration (10^{-3} M), whereas Cd inhibited callus growth at considerably lower concentrations (10^{-4} and 10^{-5} M).

The effects of lead and cadmium on callus dry matter content were also specific. At 10^{-7} M Pb, the calluses contained 6.51% of dry matter, 13% less than the control, while at 10^{-3} M Pb the dry matter content was 9.2%, or 23% higher than in the control treatment. In the case of cadmium, the only increase in relation to the control (30.7%) was recorded with 10^{-3} M — the other concentrations had no effect on callus dry matter content (Tab. 1).

Tab. 1 — Effect of different concentrations of lead and cadmium on fresh weight and dry matter content in wheat calluses

Concentration (M)	Lead		Cadmium	
	Fresh weight (mg)	Dry matter content (%)	Fresh weight (mg)	Dry matter content (%)
Control	107,0	7,47	107,0	7,47
I (10^{-7})	94,0	6,51^{-}	105,0	7,05
II (10^{-6})	134,0^{++}	6,53^{-}	117,0^{++}	6,73
III (10^{-5})	103,0	8,07	96,0^{--}	7,89
IV (10^{-4})	110,0	7,47	73,0^{--}	8,21
V (10^{-3})	61,3^{--}	9,20^{++}	14,0^{--}	9,76^{++}
LSD 0,05	13,98	0,9364	6,757	0,8632
0,01	19,33	1,2950	9,342	1,1930

Looking at the absolute values of callus dry weight (Fig. 1), it can be noticed that the differences between certain concentrations were less prominent than in the case of the dry matter content. Lead concentrations of 10^{-7} (I) and

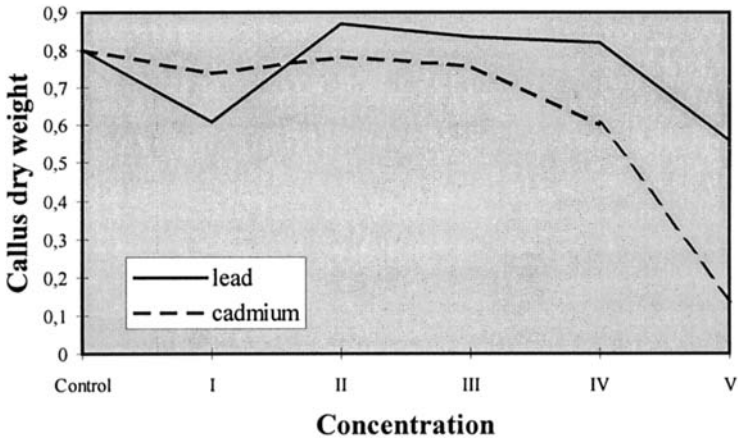

Fig. 1 — Effect of different concentrations of lead and cadmium on callus dry weight

10^{-3} M (V) reduced callus dry weight to a certain extent, while cadmium decreased the dry weight value only at the highest concentration (10^{-3} M).

The increased callus dry matter content with the highest concentrations of Pb and Cd was most likely not due to an increased synthesis of organic matter. This proposition is supported by the callus dry weight values shown in Figure 1 and the fact that they decreased at the highest concentrations of the two heavy metals. This means that the supposed rise in the callus dry matter content at the highest concentration of Pb and Cd occurred in the treatments in which calluses were considerably smaller, largely necrotic, and, in the case of Cd, dehydrated as well. Also, because these calluses had a significantly smaller weight than the control ones, the contribution of the isolated embryo (which is not separated from the callus during analysis) to total dry matter was proportionally larger than in the calluses with a larger weight obtained with the other concentrations.

The results of this study have shown that high concentrations of heavy metals inhibit callus growth. According to Kastori et al. (1997), the inhibition of plant growth at higher heavy metal concentrations occurs because these elements inhibit both the division and elongation of cells. It is assumed that cadmium causes certain changes in the cell wall that reduce its capacity for elongation and its permeability. Increased water deficit in the presence of higher lead concentrations has been reported in young sunflower plants too [Kastori et al., 1996].

In addition to the inhibitory effect of the high concentrations, we also found that the lower doses of the two metals had a stimulative effect on callus growth. Petrović and Kastori (1994) as well as Ernst (1996) also report that lower concentrations of non-essential heavy metals can have a stimulative effect on plant growth.

The reported results indicate that wheat calluses were considerably more sensitive to the presence of cadmium in the medium than to that of lead, which they were able to tolerate even at fairly high concentrations. Similar results, indicating that wheat is well able to withstand the presence of lead in the

nutrient medium, have also been reported in Diehl et al. (1983) and Kastori et al. (1991). This means that wheat tolerance to lead and its sensitivity to cadmium have also been exhibited at the level of the callus. Through this, it has been shown that calluses obtained in mature embryo culture can be used to test wheat tolerance to heavy metals, which is in agreement with the findings of Karsai et al. (1994).

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УТИЦАЈ ОЛОВА И КАДМИЈУМА НА ПОРАСТ КАЛУСА И САДРЖАЈ СУВЕ МАТЕРИЈЕ У КУЛТУРИ ЗИГОТНОГ ЕМБРИОНА ПШЕНИЦЕ (*TRITICUM AESTIVUM* L.)

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Резиме

Испитиван је утицај тешких метала олова и кадмијума на пораст калуса и садржај суве материје у калусном ткиву. Испитивано је пет концентрација олова и кадмијума: 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} и 10^{-3} М. У експерименту су коришћени калуси добијени из зрелог зиготног ембриона пшенице (*Triticum aestivum* L.) цв. Балкан. Изоловани ембриони су гајени на модификованој MS (Murashige and Skoog, 1962) хранљивој подлози. За време култивације праћен је пораст калусног ткива, да би се 30 дана након изолације приступило мерењу свеже масе калуса, као и садржају суве материје. Резултати су показали да су постојале значајне разлике између олова и кадмијума у погледу њиховог утицаја како на индукцију и пораст калуса, тако и на садржај суве материје у калусном ткиву. Највиша концентрација (10^{-3} М) и олова и кадмијума је имала значајан утицај на смањење свеже масе за 42% код олова и 87% код кадмијума, у односу на нетретирану контролу. Међутим, садржај суве материје је био повећан у поређењу са контролом и износио је 9,2% код олова и 9,8% код кадмијума, што је у поређењу са контролом повећање за 22,7%, односно 30,7%. Ефекат нижих концентрација тешких метала, које су испитиване у овом експерименту, био је знатно слабије изражен, мада је доза 10^{-6} М и олова и кадмијума имала стимулативно дејство на пораст калусног ткива.

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SILVER LINDEN (*Tilia tomentosa* Moench) IN THE COMMUNITY OF SESSILE OAK AND HORNBEAM (*Rusco-Querco-Carpinetum* B. Jov. 1979 *tilietosum tomentosae* subass. nova) ON THE FRUŠKA GORA MOUNTAIN

ABSTRACT: This paper presents the results of a phytocoenological survey performed in 27 sample locations in communities of sessile oak and hornbeam on the Fruška Gora Mountain in 1998. The visited stands are located on plateaus of broad ridges at elevations between 300 and 450 m a.s.l., in the locations of Stražilovo, Elektrovojvodina, Venac, Matijevac and Zmajevac. The dominance of silver linden within the tree and shrub layer is evidently due to a large anthropogenic influence (clear cuttings in the past). In consequence to this type of management, the silver linden has increased in numbers, mostly vegetatively, so that it is presently found in the stands in the form of brushwood which displaced sessile oaks, hornbeams and the other tree species. Taking in account the exceptional significance of the silver linden which is widespread on the margins of the Pannonian Plain, as well as its dominance in the observed stands, a new subassociation has been identified and named *Rusco-Querco-Carpinetum* B. Jov. 1979 *tilietosum tomentosae* subass. nova.

KEY WORDS: the Fruška Gora Mountain, silver linden, anthropogenic influence, *Rusco-Querco-Carpinetum* B. Jov. 1979 *tilietosum tomentosae* subass. nova.

INTRODUCTION

The silver linden is one of the most significant tree species on the Fruška Gora Montain in both scientific and practical sense. This tree species spreads over large areas from the foothills to the top of the mountain. It is present in a large number of forest communities, providing a high yield of wood mass through vegetative regeneration. Phytocoenological and ecological surveys carried out in the period from 1947 to 1956 (Janković and Mišić, 1960) had shown that during that period the silver linden was the dominant tree species

in the forests of the Fruška Gora. Clear cuttings in the past century, which continued to the present days, particularly after the World War II, resulted in the dominance of silver linden brushwood, which hinders or suppresses the regeneration of other tree species, the sessile oak in particular, by reducing the number of root suckers year after year. However, during the last half of the century, large changes occurred in the forests of the Fruška Gora, resulting in the progradation and succession of the vegetation, as reported in several papers (Mišić et al., 1997; Dinić et al., 1998). For example, differences exist in the structure and composition of species in the sessile oak-hornbeam forests of 50 years ago and the stands of the same forest type in 1998. High dominance of the silver linden in the tree layer and the shrub layer has changed the structure of the community of silver linden and hornbeam — *Rusco-Querco-Carpinetum* B. Jov. 1979 (syn. *Querco-Carpinetum aculeatetosum* B. Jov. 1951) so that the authors of this paper have defined a new subassociation with silver linden — *Rusco-Querco-Carpinetum* B. Jov. 1979 *filietosum tomentosae* subass. nova.

METHODS

The phytocoenological survey in the community of sessile oak and hornbeam with silver linden was performed after the analytical-synthetic method of Braun-Blanquet. Counts were made in 27 sample locations in the stands on the plateaus of broad ridges along the Partizanski put, at elevations from 300 to 450 m a.s.l. In order to establish the participation of the silver linden in degraded stands of sessile oak and hornbeam, sample locations were selected in the locations of Stražilovo, near Elektrovojvodina, on Venac, Matijevac and Zmajevac. Particular attention was paid to the vegetative propagation of the silver linden.

RESULTS AND DISCUSSION

The community of sessile oak and hornbeam *Rusco-Querco-Carpinetum* B. Jov. 1979 (syn. *Querco-Carpinetum aculeatetosum* B. Jov. 1951) is distributed in regions of northern Serbia, on the margin of the Pannonian Plain. In this part of Serbia, this community represents a variant of Serbian sessile oak-hornbeam community, *Querco-Carpinetum serbicum* Rudski 1949. On the Fruška Gora, the small mountain massif (539 m a.s.l.) on the southern margin of the Pannonian Plain, the community of sessile oak and hornbeam is widespread. Here it represents the basic climate-regional forest type within the belt between 300 and 500 m a.s.l. This phenomenon can be explained by the origin and historical development of the region (Bukurov, 1953, 1954), the transitional character of the climate (Milosavljević et al., 1973), the isolated, island-like, character and the low altitude of the mountain, the broken relief (Milić, 1973) and the silicate parent rock. The sessile oak-hornbeam forests are found on broad and shallow plateaus of the ridges,

broad saddles, mild northern slopes, and in shallow hollows (Dinić, 1970, 1971, 1975, 1978, 1997). They originate from „former mixed forests of the virgin forest type" (Janković and Mišić, 1960, 1980). They retained the most relict characteristics among the communities on the Fruška Gora; they are richest in relict species from south and east (Obradović, 1966). The presence of relict sub-Mediterranean species *Ruscus aculeatus* and *Ruscus hypoglossum* is an indication of not only a favorable microclimate of the forests but also of the generally prevailing climatic and edaphic conditions (Jovanović, 1967, 1985). Already 50 years ago, as well at today, it was possible to find stands of sessile oak and hornbeam on the Fruška Gora containing more then 10 other tree species. Those were the remnants of the former mixed forests that grew on this massif as well as in other parts of Serbia (Mišić, 1982, 1994).

During last 50 years, the silver linden (*Tilia tomentosa* Moench) increased its numbers on the Fruška Gora, participating in this way in the succession of the sessile oak forests on this mountain. Recent surveys of sessile oak stands on the Fruška Gora (Mišić et al., 1997; Dinić et al., 1998) showed that silver lindens penetrated the pure sessile oak forests of the type *Festuco drymeiae-Quercetum petraeae* (Jank. et Miš. 1960) Jank. 1968, gradually forming, in the process of progressive succession, the community *Tilio tomentosae-Quercetum petraeae* Dinić, Mišić et Savić 1998, in which *Festuca drymeia* is rarely found. The situation with the participation of the silver linden in the forming of the community *Rusco-Querco-Carpinetum* B. Jov. 1979, will be seen from the recent phytocoenological surveys from Stražilovo to Zmajevac. The aim of this paper was to compare the sample locations of 50 years ago with those from 1998, in order to attest the gradual penetration of the silver linden into the sessile oak-hornbeam forests on broad plateaus of the Fruška Gora Mountain. At the same time we want to demonstrate how the numbers of the sessile oak, the main edifier in these forests, are being gradually reduced, causing changes in the structure and aspect of the sessile oak--hornbeam community.

In the sessile oak-hornbeam stands on the Fruška Gora Mountain surveyed 50 years ago, among the 8 species recorded in the tree layer, the dominant species was the hornbeam (*Carpinus betulus*) with the numbers and cover degree of 2.2 to 4.3 (Janković and Mišić, 1960). In the same phytocoenological table (page 50), in the tree layer, the hornbeam was followed by the sessile oak (*Quercus petraea*), with the numbers and cover degree of +.1 to 3.2, and the silver linden (*Tilia tomentosa*) with the numbers and cover degree of r.+ to 3.2. It was characteristic that the silver linden occurred only in two sample locations with the numbers and cover degree of 2.2 to 3.2, while in the other locations its numbers were considerably lower. It means that, at that time, the silver linden had not managed to occupy the layers of higher and lower trees. Among the 24 species in the shrub layer, the dominant species was the hornbeam, followed by the silver linden, the numbers of which in the stand were very low. Analyzing the growth of young trees in the herb layer, not a single silver linden individual was found among the 13 species present (Janković and Mišić, 1960). Obviously, the silver linden has poor gener-

ative regeneration, as confirmed by the recent phytocoenological survey in the sessile oak-hornbeam community.

The analysis of the phytocoenological table (Table 1), containing data recorded in 27 sample locations established from Stražilovo to Zmajevac on the Fruška Gora in 1998, has shown that 13 species were present in the tree layer and 25 species in the shrub layer. The silver linden was dominant in the analyzed stands with the numbers and cover degree of 3.3 to 5.4. The hornbeam occurred in the tree layer with the numbers and cover degree lower by a half, 1.1 to 4.3, followed by the sessile oak with the values of r to 4.2. Obviously, the silver linden has pushed back the hornbeam and particularly the sessile oak. There are stands on Stražilovo (Table 1, locations 5, 6, 7, 8) without hornbeams in the tree layer, as a result of poorer regeneration of this species in habitats predominated by the silver linden. The numbers of the other species are also reduced in these forests. On Venac, Matijevac and Zmajevac, the structure of the communities of sessile oak and hornbeam was more favorable, as evidenced by a larger number of species in the tree layer. In the nature preserve at Zmajevac, for example, 8—10 tree species were found in the tree layer (Table 1, locations 26, 27). The stands on Zmajevac demonstrate what these forests would have looked like had there been no human influence.

Greatest changes have taken place in the stands of sessile oak and hornbeam near Elektrovojvodina. It should be mentioned that in some stands the sessile oak has been completely extinct or is represented by a few trees (Table 1, locations 12, 13, 16, 17). In this location, the silver linden was dominant in all investigated stands. In the tree layer, individual silver lindens that were spaced wide apart developed brushwood containing 5 stems (Figure 1). In younger stands, silver linden brushwood was found with 10 to 15 suckers emerging from one stump (Figure 2). In almost all investigated stands we have encountered „sucker nests" of silver linden around fully-grown trees (Figures 3 and 4), resulting from the cutting of individual trees and subsequent vegetative regeneration from stumps and roots. After entering such forests, we had the impression that these silver linden shrubs emerged from seed. In the stands near Elektrovojvodina, sessile oak brushwood with three to four stems could be seen (Figure 5) and this is also the result of previous clear cutting in these habitats.

Such human behavior in the past has changed the aspect of the former rich sessile oak-hornbeam forests. They have been transformed into degraded stands predominated by the silver linden (Figures 6 and 7). Presently on Stražilovo, there are vast clearings (Figure 8) in which the butcher's broom (*Ruscus aculeatus* L.) predominates in the shrub layer and the herb layer (Table 1, locations 5, 6). The dominance of the silver linden in the tree and shrub layer, as well as the reduced number of sessile oaks and hornbeams in the investigated stands, resulted from clear cuttings practiced in recent and distant past. After a tree is felled, a cluster with several suckers develops in a few years; some of the suckers can achieve the height and diameter of a tree grown from seed. After the next cutting, the newly emerged suckers are shorter and they have a smaller diameter.

Fig. 1 — Silver linden (*Tilia tomentosa* M o e n s c h) brushwood in a stand of the community of sessile oak and hornbeam near Elektrovojvodina on the Fruška Gora Mountain (Photo: Šandor Lukač, Novi Sad)

Fig. 2 — Young suckers from a silver linden (*Tilia tomentosa* M o e n s c h) stump in a stand of the community of sessile oak and hornbeam near Elektrovojvodina on the Fruška Gora Mountain (Photo: Šandor Lukač, Novi Sad)

Fig. 3 — A brushwood of suckers from a silver linden (*Tilia tomentosa* M o e n s c h) stump near mature silver linden trees growing in a stand of the community of sessile oak and hornbeam near Elektrovojvodina on the Fruška Gora Mountain (Photo: Šandor Lukač, Novi Sad)

Fig. 4 — A brushwood of suckers from a silver linden (*Tilia tomentosa* M o e n s c h) stump near mature silver linden trees growing in an open stand of the community of sessile oak and hornbeam near Elektrovojvodina on the Fruška Gora Mountain (Photo: Šandor Lukač, Novi Sad)

Fig. 5 — Sessile oak (*Quercus petraea* /Matt./Liebl.) brushwood in a stand of the community of sessile oak and hornbeam in the location of Stražilovo on the Fruška Gora Mountain (Photo: Šandor Lukač, Novi Sad)

Fig. 6 — A stand of the community *Rusco-Querco-Carpinetum tilietosum tomentosae* subass. nova in the location of Elektrovojvodina on the Fruška Gora Mountain (Photo: Šandor Lukač, Novi Sad)

Fig. 7 — A stand of the community *Rusco-Querco-Carpinetum tilietosum tomentosae* subass. nova on the Fruška Gora Mountain (Photo: Šandor Lukač, Novi Sad)

Fig. 8 — Butcher's broom (*Ruscus aculeatus* L.) in an open stand of sessile oak and hornbeam in the location of Stražilovo on the Fruška Gora Mountain (Photo: Šandor Lukač, Novi Sad)

The 1998 phytocoenological survey showed that silver linden saplings seldom occur in the herb layer (Table 1). The same observation was made in the sessile oak-hornbeam stands 50 years ago (Janković and Mišić, 1960). Although the structure of the community of sessile oak and hornbeam has been considerably changed by the dominance of the silver linden, the changes in the herb layer have not reached the corresponding extent. The openings made in many of the investigated stands increased the numbers and cover degree of some species such as *Hedera helix, Rubus hirtus, Galeobdolon luteum*, and *Ruscus aculeatus* (Table 1). *Rubus hirtus* has become a serious problem in these stands, because the sessile oak regeneration from seed unfolds poorly in the understory of this species. The survey of L. Đurđević (1989) showed that the blackberry, more than the other species in the community, exude choline which accumulates in the soil in quantities inhibitory for seed germination.

The complete analysis of the plant species composition enabled us to identify a new subassociation with the silver linden — *Rusco-Querco-Carpinetum* B. Jov. 1979 *tilietosum tomentosae* subass. nova, predominated by mesophytic species from the alliance *Carpinion betuli moesiacum* B. Jov. 1986 and the order *Fagetalia sylvaticae* Pawl. 1928 (Dinić, 1997; Tomić, 1992).

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СРЕБРНА ЛИПА (*TILIA TOMENTOSA* MOENCH) У ЗАЈЕДНИЦИ КИТЊАКА и ГРАБА (*RUSCO-QUERCO-CARPINETUM* B. JOV. 1979 *TILIETOSUM TOMENTOSAE* SUBASS. NOVA) НА ФРУШКОЈ ГОРИ

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Резиме

Сребрна липа (*Tilia tomentosa* Moench) заузима велике површине од подножја до врха Фрушке горе. Највеће учешће има у китњаковим, китњаково-грабовим и буковим шумама. Честе сече у прошлом и овом веку, а посебно после Другог светског рата, довеле су до доминације липе у бокорима, која је отежавала и онемогућавала подмлађивање и раст других врста дрвећа, а нарочито китњака, који је из године у годину смањивао бројност својих јединки. Последњих пола века десиле су се крупне промене у шумама Фрушке горе, које су резултат прогресивне сукцесије вегетације. Тако, на пример, постоје разлике у структури између заједнице китњака и граба од пре 50 година и садашњих састојина истог типа шуме снимљених 1998. године. У овом раду приказујемо резултате фитоце-

нолошких испитивања на Фрушкој гори у заједници китњака и граба на платоима широких гребена на висини од 300 до 450 m н.в. Узето је 27 фитоценолошких снимака на локалитетима: Стражилово, Електровојводина, Венац, Матијевац и Змајевац. У свим испитиваним састојинама констатована је доминација сребрне липе у спрату дрвећа и жбунова, што је последица великог антропогеног утицаја. Сребрна липа је повећала бројност својих изданака претежно вегетативним путем, тако да се сада у састојинама налази у бокорима потискујући китњак, граб и остале врсте дрвећа. У овим састојинама врло ретко се могу срести жбунови и младице семеног порекла. Због изузетног значаја сребрне липе, која је широко распрострањена на ободу Панонске низије, и њене доминације у овим састојинама, издвојена је субасоцијација: *Rusco-Querco-Carpinetum* B. Jov. 1979 *tilietosum tomentosae* subass. nova.

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EFFECTS OF SOME BIOTIC FACTORS ON THE BIOLOGICAL VALUE OF SUGAR BEET SEED (*BETA VULGARIS* L.)

ABSTRACT: A three-year trial was carried out to determine the effects of plant number (stand density) and harvesting date on seed vigor, seed viability, and seed fill in sugar beet. Neither of the two factors had any significant effect on the qualitative characteristics of sugar beet seeds, although the seed did have a somewhat higher viability when sown at a spacing of 50 x 12 cm. At the later harvesting dates, the proportions of filled and viable seeds was such as to provide high quality seeds after further processing.

KEY WORDS: seed vigor, seed viability, seed fill, harvesting dates, plant number.

INTRODUCTION

Many authors have studied the effects of environmental factors and cultural practices on seed quality. Bornscheuer (1972) argues that conditions under which seed is produced significantly determine its quality. In a study by Pendelton (1954), the viability of sugar beet seeds was not affected by the change of row-to-row spacing from 30 to 60 cm and the effect of changing plant-to-plant spacing from 4 to 16 cm was only slight. In Csapody (1984), watering proved the key determinant of seed quality relative to non-watered treatments. Similar results were obtained by Horvath (1984), who found that non-watered treatments had twice as many empty seeds as those that were watered.

Most researchers point to premature harvesting as the main reason for reduced seed viability. According to Inoue and Yamamoto (1977), the levels of germination inhibitors in the seed decrease as the plant matures. Similarly, Kastori (1984) states that because the seed contains germination-inhibiting substances sugar beet seeds often germinate with difficulty. The existence of such inhibitors is confirmed by the fact that when they are removed from the seed by immersion in water or rinsing out, the seed will germinate fast. Longden (1973) has established that seed viability peaks two weeks

after the reaching of maximum yields. Battle and Whittington (1969) reports significant influence of harvesting date on seed emergence. The loss of seed due to overripeness after the optimum harvesting date takes place at a pace of 10% a week (Longden, 1972). Podlaski considers seed color a more reliable indicator of maturity than either seed moisture content or the ease with which the seed can be separated from the branch.

The objective of this paper was to determine the effects of plant number (stand density) and harvesting date on seed quality in sugar beet.

MATERIALS AND METHODS

The field trial was conducted during 1996, 1997, and 1998 at Sivac. Harvesting date was Factor A: harvesting date

1) 30 days after full flowering
2) 40 days after full flowering
3) 45 days after full flowering
4) 50 days after full flowering

Factor B: plant number

1) 50 x 9 cm
2) 50 x 12 cm
3) 50 x 17 cm
4) 50 x 25 cm

The trial was set up in the form of stripe-like plots as opposed to square ones and the basic plot size was 10 m^2.

RESULTS

Vertical axes in Figure 1 show the seed vigor value for each spacing from the study. At the beginning of the growing season, seed vigor was 21% lower compared with overall seed viability. On the second, third, and fourth harvesting dates, the differences were 15, 5, and 1%, respectively. As the seed matured, germination rate increased both in absolute terms and relative the overall seed viability. Plant spacing had no effect on seed vigor.

In practice, sugar beet is harvested sometime between the second and third harvesting dates based on plant aspect. In our trial, when seed viability reached 73%, it kept increasing until the third harvesting date (Fig. 1). Between the third and fourth dates, during maturation, there was no increase in the viability of the seed. Between the first and third date, viability increased by 32%. This shows that early harvesting may reduce seed viability, which after the third harvest date no longer increases.

Stand density did not affect seed viability (Fig. 2), i.e., plant spacing had no effect on how fast physiological maturity or viability were reached. Seed viability was somewhat higher at the 50 x 12 cm spacing and somewhat lower

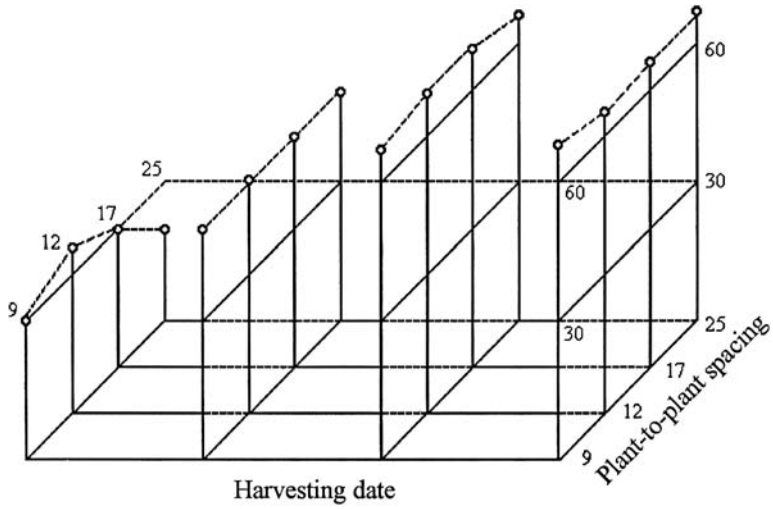

Fig. 1 — Seed vigor in sugar beet — three-year average

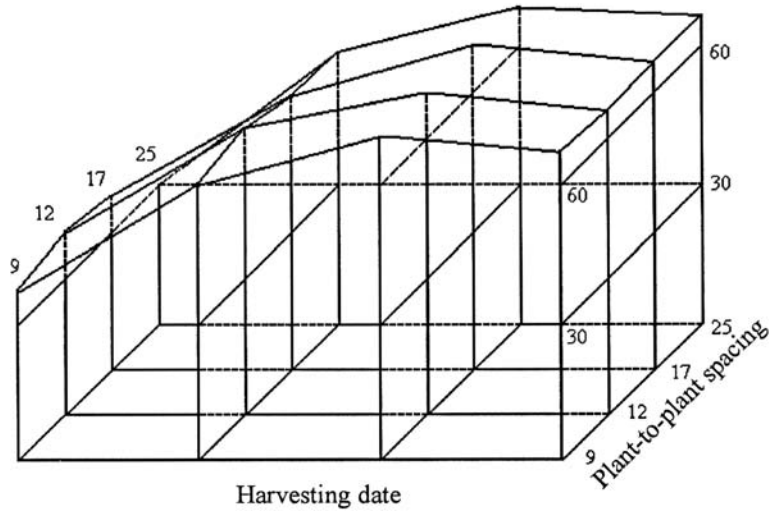

Fig. 2 — Seed viability in sugar beet — three-year average

at 50 x 24 cm, but the differences never exceeded the 5% threshold of significance.

The average seed fill levels over the three-year period for all four harvesting dates and plant spacings are marked with dotted lines in Figure 3. The concept of the average level of seed fill underlines an important difference between filled and viable seeds. If this difference is larger than 7—9%, processing will not be able to produce the 90% level of viability needed for sowing. In the third and fourth harvesting dates, the ratio of filled to viable seeds was sufficient to provide a high percentage of viable seeds for sowing.

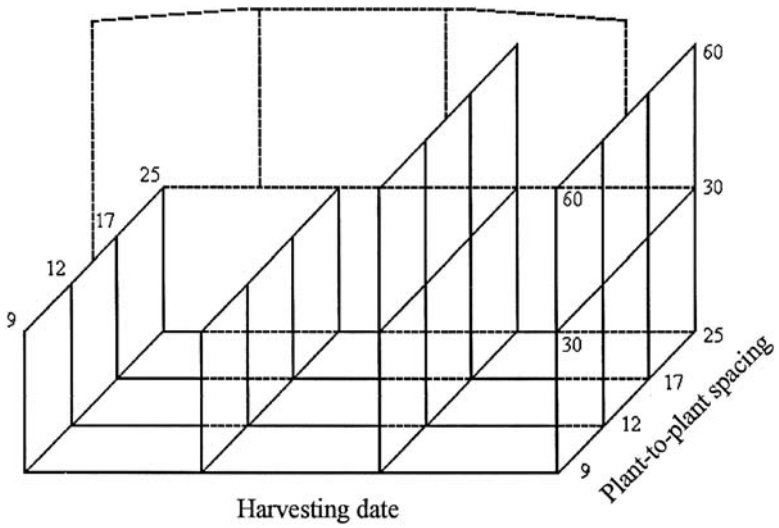

Fig. 3 — Seed fill level in sugar beet — three-year average

DISCUSSION

Viability is the most important qualitative character of seeds sown in the field, hence the need to determine seed vigor. Akeson and Widner (1980) conducted a study to compare emergence in field conditions with germination in the laboratory and in sand. The standard laboratory test was shown to be unreliable for field emergence, while the correlation of germination in sand proved to be high — r = 0.89—0.98. Similar results were obtained by Herzog and Rober (1983), who compared different methods for germination in the laboratory with field emergence and obtained the highest correlation with germination percentage in the laboratory. Longden (1972), too, reported a high correlation (r = 0.96), while Durant et al. (1985) obtained a somewhat lower correlation value — r = 0.88. All these data show germination percentage determined under controlled conditions to be the best indicator of seed's agricultural value.

Over the three years of our study, seed viability exhibited a certain regularity. The effect of plant spacing was small, except for the first harvesting date. Seed viability was better on harvesting dates used in large-scale production with a spacing of 50 x 12 cm. Optimum maturity was attained on the second and third harvesting dates and the proportion of empty seeds was 20—30%. During the trial years, vegetative development was favorable, so the plants had a high pollination percentage. Because of high temperatures in June and July, a large number of fertilized flowers produced a high percentage of empty seeds. This percentage increased on later harvesting dates. The falling off of large filled seeds due to overriperness (which is typical of seed sugar beet) also contributed to this.

The seed fill level is an important qualitative characteristic of sugar beet seed. In the present study, the level of seed fill increased until the third harvesting date. Plant spacing had no effect on this character, either. There is an explanation for this, since the stand densities used in the study fall within the normal plant population range. If to this we add the fact that the weather conditions varied according to the year, the great importance of choosing the right harvesting date for seed sugar beet becomes apparent. Stefanović (1987) obtained the highest seed viability with a plant-to-plant spacing of 12—16 cm. In addition to this, the monitoring of seed fill level and seed viability was not conducted according to the phenophases in different climatic years. The harvesting dates in different years differed by as much as 10 days in some instances.

Seed fill and vitality increased until the third harvesting date. If all of the seeds had been filled, the seed viability on the first, second, and third harvesting dates would have been 42, 68, and over 90%, respectively. According to Snyder (1971), seed with a 90% viability level can be considered physiologically mature. The three-year average for the seed in our study to reach the 90% viability level was 50 days, and the seed moisture content was 50%. Similar results are reported by Te Krony (1969), who obtained the highest viability 43 days after full pollination. In Stefanović (1987), seed maturity increased until the second harvesting date, i.e., 37—44 days after full flowering. In practice, the most reliable method to determine maturity is to observe plant color and the appearance of the endosperm. Longden and Johnson (1984) state that seeds can germinate 15—40 days after flowering.

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УТИЦАЈ НЕКИХ БИОТИЧКИХ ЧИНИЛАЦА НА БИОЛОШКУ ВРЕДНОСТ СЕМЕНА ШЕЋЕРНЕ РЕПЕ (*BETA VULGARIS* L.)

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Резиме

Пољским трогодишњим огледом хтели смо да одредимо утицај броја биљака и рока жетве на енергију клијања, клијавост семена и испуњеност семена шећерне репе. Утицаја примењене мере неге и времена жетве није било. Остварена је већа клијавост семена код размака биљака 50 x 12 cm, али без значајних разлика у одосу на остале размаке између биљака. Повећање клијавости било је 32% до трећег рока жетве. Из тога се може закључити да се раном жетвом смањује клијавост семена шећерне репе. Просечна испуњеност семена је разлика између испуњених и клијавих плодова. Ако је разлика већа од 9%, дорадом семена не може се добити клијавост семена за сетву преко 90%.

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ALLOZYME VARIABILITY IN THE NATURAL POPULATION OF HARES (*Lepus europaeus* Pallas)

ABSTRACT: The isozyme variability of the hare population in the Vojvodina Province has been analyzed in order to establish genetic diversity needed to identify a genotype that may be successfully adapted to the deteriorated eco1ogical conditions. Analyses were done by the polyacrylamide gel e1ectrophoresis method, investigating the variability in 21 loci. The obtained results show that only *Mdh-1, Idh-2, 6-Pgd-2, 6-Pgd-3, Pgm-2, Est-2, Est-3, Est-4, Est-5* and *Est-6* were polymorphic, possessing two to six alleles. The value of average heterozygosity (H) was 0.145, while polymorphism (P) was 0.295.

KEY WORDS: allozyme, the brown hare (*Lepus europaeus*), genetic identity, genetic variation

INTRODUCTION

During last three decades, a sudden drop occurred in the hare populations in the Vojvodina Province and the entire Europe (Šelmić, 1980; Šelmić, 1997; Vapa and Šelmić, 1997). Causes of this phenomenon have not been investigated in full detail. Some risk factors are relatively well known (climatic factors, intensive agriculture, various diseases, hunting pressure, etc.); others may be consequences of reduced genetic variability, which unfavorably affects other characteristics, similarly to the situation with the domestic animals (decreased reproductive efficiency and stress resistance, occurrence of diseases, etc.).

Genetic variability is considered a starting point for evolutionary trends, but it is also an indication of the changes that had occurred in the past (Weir, 1990). The importance of investigating the wild animals' genome is emphasized by the fact that a part of the genome is shared with their domestic relatives. Progress in plant and animal production is based on genetic diversity. Today it is widely accepted that the conservation of genetic diversity increases chances of animal survival. It has also been recognized that low gene-

tic variability is associated with inbreeding depression and loss of heterozygosity, weakening the components of the population phenotype including metabolic efficiency, increase intensity, reproductive efficiency, disease resistance, etc. (Gilpin and Soule, 1986). Hartl et al. (1992) reported of better chances for survival of heterozygous females, which may significantly improve the genetic structure of the population. In wild animals, the risk of genetic variability decrease may be even greater, in consequence to small populations, long-term cognate breeding, selective hunting for trophies, etc. Disappearance of certain genes significantly reduces chances for genetic combinations, i.e., new genetic variability, to occur some day, producing individuals that are better adapted to the changes in the environmental conditions and the new agricultural technologies. Therefore, the main objective of conservative biology is the preservation of genetic variability within the wild animals' population, in order to avoid the unfavorable effects of long-term cognate breeding (inbreeding depression).

Investigations of genetic variability in wild animals, especially in the brown hare, have been relatively scarce (Hartl et al., 1989, 1990, 1992, 1993; Vapa et al., 1994, 1995, 1997; Suchentrunk et al., 1998, 1999); therefore, every contribution in this sense is extremely important.

Presently it is possible to analyze the animal genome by molecular genetic methods, i.e., by using genetic markers. Distinguishing characteristics of genetic markers are that they are present from the time of birth, that they remain unchanged by environmental factors (diet, climatic factors, diseases, etc.) during the entire life span, that they are inherited ca-dominantly and that they can he objectively proven. The most frequently used genetic markers are isozymes which occur in several molecular forms, the so-called allozymes.

The aim of this study was to establish the frequency of isozyme alleles and to calculate parameters of genetic variability — heterozygosity (H), polymorphism (P), genetic identity (I) and genetic distance (D) in several local populations of the brown hare.

MATERIAL AND METHODS

Brown hares were collected in four locations in the Vojvodina Province: Banatsko Aranđelovo, Begeč, Despotovo and Pačir. All animals were obtained during hunts in the winter of 1998, sexed and aged. Liver samples were frozen in liquid nitrogen immediately after death of the animals, and stored at —20°C until electrophoresis. Tissue was macerated at 4°C in TC stock buffer pH 7.1 (containing Tris 0.8M, citric acid 0.24M, sucrose 10%, Triton X-100 0.1%, Bromophenol Blue 0.1% in 100 ml H_2O), at the ratio 1:10 (w/v). After centrifugation for 3 minutes at 4°C and 16,000 rpm, 1.5 to 15 μ1 (depending on enzyme) of clear supernatant were placed in gel slots.

The following nine enzyme systems were screened:

1. lactate dehydrogenase (LDH, E.C. 1.1.1.27)
2. 3-hydroxibutirat dehydrogenase (HBD, E.C. 1.1.1.30)
3. malate dehydrogenase (MDH, E.C. 1.1.1.37)

4. malic enzyme (ME, E.C. 1.1.1.40)
5. isocitrate dehydrogenase (IDH, E.C. 1.1.1.42)
6. 6-phospho gluconate dehydrogenase (6-PGD, E.C. 1.1.1.44)
7. superoxide dismutase (SOD, E.C. 1.15.1.1)
8. phosphoglucomutase (PGM, E.C. 2.7.5.1)
9. esterase (EST, E.C. 3.1.1.1)

Vertical polyacrylamide gel electrophoresis was performed in a LKB system at 4°C, in:

— TBE buffer system pH 8.9 (0.08M TRIS, 0.0015M EDTA, 0.02M boric acid) was used fur MDH, ME, 6-PGD, PGM and EST), with constant 300V.

— TC buffer system pH 7.1 (0.02M TRIS, 0.006M citric acid) was used for LDH, HBD, IDH and SOD), with constant 221V.

After electrophoresis, gels were stained according to Selander et al. (1971).

The values of polymorphism and heterozygosity were calculated according to Ayala et al. (1975), genetic identity (I), distance (D) and UPGMA dendrogram were done after Nei (1972).

RESULTS AND DISCUSSION

The screening for the nine enzyme systems represented by twenty-one presumptive structural loci revealed polymorphism in only five enzymes at ten loci in the population of the brown hare.

The loci for *Ldh-1* and *Ldh-2*, *Hbd-1* and *Hbd-2*, *Mdh-2*, *Me-1*, *Idh-1*, *6-Pgd-1*, *Sod-1* and *Pgm-1* were monomorphic, the loci for *Mdh-1*, *Idh-2*, *6-Pgd-2*, *6-Pgd-3*, *Pgm-2*, *Est-2*, *Est-3*, *Est-4* and *Est-5* were polymorphic, showing two to six different alleles (Table 1). Hartl et al. (1990, 1992), Vapa et al. (1994) and Suchentrunk et al. (1999) described the polymorphism in the loci for Ldh-2, Idh-2, Pgm-2, Mdh-2, 6-Pgd and Est from different hare tissues, but the highest polymorphism was found in liver tissue (Vapa et al., 1994).

Tab. 1 — Allele frequencies at the polymorphic loci in the brown hare population

Locus	Despotovo	Begeč	B. Aranđelovo	Pačir	Mean
Mdh-1	1.0	0.4	0.66	1.0	0.765
	0.0	0.6	0.33	0.0	0.232
Idh-2	0.875	0.5	0.66	1.0	0.758
	0.125	0.5	0.33	0.0	0.238
6-Pgd-2	0.25	0.4	0.0	0.2	0.212
	0.75	0.6	1.0	0.8	0.787
6-Pgd-3	0.875	1.0	1.0	1.0	0.968
	0.125	0.0	0.0	0.0	0.031
Pgm-2	0.875	0.9	1.0	0.0	0.056
	0.125	0.1	0.0	0.0	0.025

Est-1	0.0	0.1	0.0	0.0	0.025
	1.0	0.9	1.0	1.0	0.975
Est-2	0.0	0.0	0.166	0.0	0.041
	0.0	0.0	0.166	0.0	0.041
	0.25	0.4	0.0	0.2	0.212
	0.25	0.0	0.166	0.1	0.129
	0.25	0.1	0.33	0.1	0.195
	0.25	0.5	0.166	0.6	0.379
Est-3	0.0	0.1	0.0	0.0	0.025
	0.0	0.4	0.5	0.0	0.225
	0.0	0.1	0.0	0.0	0.025
	1.0	0.4	0.5	1.0	0.725
Est-4	0.0	0.2	0.0	0.0	0.05
	0.375	0.7	0.33	0.5	0.476
	0.25	0.0	0.166	0.0	0.104
	0.375	0.1	0.5	0.5	0.368
Est-5	0.125	0.5	0.5	0.0	0.281
	0.875	0.5	0.5	1.0	0.718

Means of the observed single locus heterozygosities were up to 0.74 (Table 2). The values of allozyme variability based on all loci were 0.145 for average heterozygosity (H), and 0.25 for polymorphism (P). The data for H and P obtained in this paper are similar to those of Selander (1976), but higher than those reported by Hartl et al. (1992). Studying the variability at 39 loci, of which 8 were polymorphic, in 193 hare specimens, they obtained the values of H = 4,7% and P = 18%. These differences may be due to the fact that different allozymes were investigated, to the significantly lower number of individuals analyzed in this paper, but primarily to the use of the polyacrylamide gel electrophoresis (as opposed to the starch gel electrophoresis used by Hartl et al. 1992), which provides more precise data and enables the detection of a larger number of alleles.

Tab. 2 — Heterozygosity and polymorphism in the brown hare population

Locus	Despotovo	Begeč	B. Aranđelovo	Pačir	Mean
Ldh-1	0.0	0.0	0.0	0.0	0.0
Ldh-2	0.0	0.0	0.0	0.0	0.0
Hbd-1	0.0	0.0	0.0	0.0	0.0
Hbd-2	0.0	0.0	0.0	0.0	0.0
Mdh-1	0.0	0.0	0.0	0.0	0.0
Mdh-2	0.0	0.0	0.0	0.0	0.0
Me-1	0.0	0.0	0.0	0.0	0.0
Idh-1	0.0	0.0	0.0	0.0	0.0
Idh-2	0.25	1.0	0.66	0.0	0.477
6-Pgd-1	0.0	0.0	0.0	0.0	0.0

6-Pgd-2	0.0	0.0	0.0	0.0	0.0
6-Pgd-3	0.25	0.0	0.0	0.0	0.062
Sod-1	0.0	0.0	0.0	0.0	0.0
Pgm-1	0.0	0.0	0.0	0.0	0.0
Pgm-2	0.25	0.2	0.0	0.0	0.112
Est-1	0.0	0.2	0.0	0.0	0.05
Est-2	0.5	0.2	1.0	0.6	0.575
Est-3	0.0	1.0	1.0	0.0	0.5
Est-4	0.75	0.2	1.0	1.0	0.737
Est-5	0.25	1.0	1.0	0.0	0.562
Est-6	0.0	0.0	0.0	0.0	0.0
Mean H	0.107	0.18	0.22	0.076	0.145
Mean P	0.33	0.43	0.28	0.14	0.295

The allele frequencies at 10 polymorphic loci were used for the calculation of Nei's values of genetic identity (I) and distance within the hare population (Table 3). The highest value of genetic identity was found between the hares from Despotovo and Pačir (0.98), the lowest between the hares from all locations in comparison with those from Begeč (0.92) (Table 3). Apart from the relatively high values of polymorphism and heterozygosity, it may be concluded that the level of genetic variability is low (especially visible in the dendrogram), being at the level of different populations of the same species (Ayala et al., 1975).

Tab. 3 — Genetic identities (above diagonal) and genetic distances (below diagonal) in the brown hare population

Populations	Despotovo	Begeč	B. Aranđelovo	Pačir
Despotovo	—	0.92	0.95	0.98
Begeč	0.08	—	0.93	0.91
B. Aranđelovo	0.05	0.07	—	0.93
Pačir	0.02	0.09	0.07	—

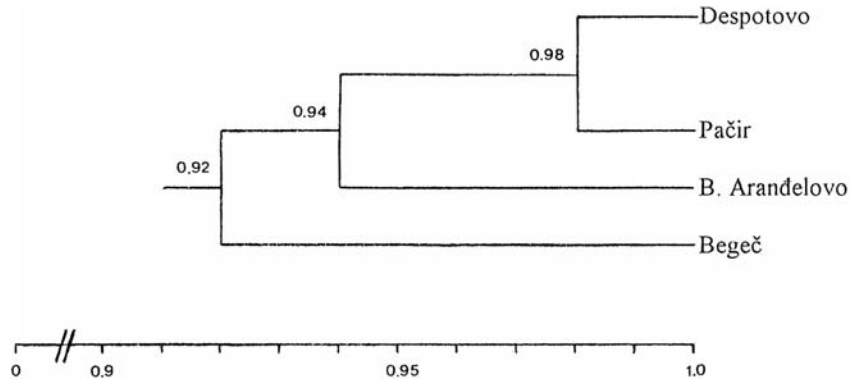

Fig. 1 — UPGMA dendogram summarizing the genetic identity of the hare population

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ВАРИЈАБИЛНОСТ АЛОЗИМА У ПОПУЛАЦИЈИ ЗЕЦА (*Lepus europaeus* Pallas)

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Резиме

У раду је анализирана варијабилност изозима популације зеца Војводине, у циљу утврђивања генетичке разноврсности, што би могло указати на генотип који је погоднији за успешну адаптацију ове врсте у природи. Анализе су рађене методом полиакриламид-гел-електрофорезе, а испитана је варијабилност двадесет и једног ензимског локуса. Резултати су показали да су само локуси *Mdh-1, Idh-2, 6-Pgd-2, 6-Pgd-3, Pgm-2, Est-2, Est-3, Est-4* и *Est-5* били полиморфни и поседовали два до шест различитих алела. Вредност просечне хетерозиготности (H) је била 0.145, док је полиморфност (P) износила 0.295. И поред релативно високих вредности полиморфности и хетерозиготности испитиваних алозима може се закључити да је степен генетичке варијабилности низак и на нивоу је различитих популација исте врсте.

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RADIOLOGICAL FINDINGS IN PATIENTS WITH COMPLETE DENTURE

ABSTRACT: Diagnostic profit from a radiographic survey of complete-denture patients is considerable, even when clinically there is no evidence of pathology. The earlier opinion that all retained roots or root fragments sooner or later become infected has been abandoned. In a sample of 110 patients who used complete dentures for five or more years, 22.72% of them had retained roots. Retained toots were found in 4.55% of the total number of radiograms. On the basis of our results it can be concluded that retained roots and unerupted teeth, in the absence of clinical symptoms or radiographic evidence of enlarged follicular sacs or on evidence of resorption, need not be removed from the edentulous mouth.

KEY WORDS: edentulism, retained roots, unerupted teeth

INTRODUCTION

Although it has been concluded in many studies that diagnostic profit from a radiographic survey of complete-denture patients before denture therapy is great, even when there is no clinical evidence of pathology, this type of examination has not yet become a standard practice.

A possible reason for this is the reluctance to subject the patient to ionizing radiation, because of the substantial quantity of radiation and real negative effects. However, in a report of Keur (K e u r et a1., 1986), the decision to make a radiographic examination rests upon a professional judgment of the benefits which accrue to the total health of the patient as opposed to any biological effects which might be caused by the radiation.

There is general agreement in the dental literature that the main hazard in dental radiography is a possibility of inducing carcinomatous change in the directly irradiated radiosensitive organs within the head and neck region. Current opinion is that the total cancer risk per million ortopantomograms is 1—4 cases (K e u r et al., 1986).

The second reason can be the opinion of dentists that preliminary panoramic radiography of the edentulous mouth is not necessary. This assertion is in

agreement with the result of White (White, Weisman, 1977) who found that the treatment plan was altered in only 7% of patients as a result of the radiographic observation of retained root fragments or unerupted teeth.

Pathologic findings from a technically good radiograph of the edentulous mouth are: retained tooth fragments, impacted teeth, pathology of the temporomandibular joints and maxillary antra, radiolucencies and radiopacities, elongated styloid processes, calcification of lymph nodes and arteries, foreign bodies (dental amalgam and ligature fixation wires) and abnormal positioning of the mental foramen relative to the mandibular ridge crest.

The question remains open as to the kinds of changes that necessitate surgical intervention. The earlier opinion that all retained roots or root fragments sooner or later become infected (Garcia et al., 1987) is no longer accepted.

Herd (Keur et al., 1986), correlating radiological findings of retained tooth roots with histological findings, found that nearly 73% of all roots without previous radiographic evidence of periradicular pathology displayed a similar histological pattern. Based upon this evidence, the authors concluded that fragments that do not show any clinical and radiographic abnormality could be considered to have been accepted by the surrounding tissues.

Mastication forces that pass via denture base onto supporting tissues influence greatly the dynamism of the bone. Thus it is necessary to define the kind of retained fragments present and their location in the bone which make surgical intervention necessary.

The aim of the present study was to investigate the kind and position of retained roots and teeth that do not necessitate extraction from the edentulous mouth.

MATERIAL AND METHODS

A sample of 110 fully edentulous patients on the waiting list for a new complete denture at the Dental Clinic, Medical Faculty, University of Novi Sad, was examined clinically and radiographically. All patients had previously used complete dentures for five years or longer. The study included patients in whom no clinical symptoms could be detected by inspection and palpation. Ortopantomograms were exposed at 65—70 kV and 225 mA on an Ortopantomograph 3, Siemens AG.

Results that were looked for were retained roots and impacted teeth. The kind, number and location of teeth or tooth fragments were registered in regard to the kind of the jaw and the maxillary or mandibulary side. Perifocal inflammation was registered when a radiolucent region was visible on the radiograph. If, after an initial viewing of the orthopantomogram, any area on the radiograms was not sufficiently clear or if pathology was suspected, additional views by periapical radiograms were taken.

RESULTS

Of the 110 patients included in the survey, 65 (59.09%) were females and 45 (40.90%) were males. Forty-eight patients (43.63%) were above the age of 65.

Abnormalities such as *rubber, decubitus* or *hyperphlasio mucosae* and subjective symptoms were not found.

Table 1 shows the numbers, percentages, and distribution between sexes of the patients with positive findings. It was found that 11 women (10% of the total number of patients) and 14 men (12.72%) possessed retained roots. The total number of patients with retained roots was 25 or 22.72%. Retained teeth were found in 4.55% of the radiograms. One patient had three retained wisdom teeth, three patients had two retained teeth and one patient had one retained tooth. In all of these cases, the retained teeth were invariably third molars. There was no evidence of pathology in a single radiogram.

Tab. 1 — Distribution of positive radiographic findings by sex

Sex	Retained roots			Retained teeth		
	N	n	%	N	n	%
Male	45	14	12.72	45	5	4.55
Female	65	11	10	65	0	0
Total	110	25	22.72	110	5	4.55

There appears to be no jaw preference for retained teeth, although the findings of retained roots were higher in *mandibula* (15.45% of the patients) than in *maxillae* (7.27% of the patients). It can also be seen that all of the retained teeth were on the right side of the mandible, but in the upper jaw there was no side preference (Table 2).

Tab. 2 — Number and distribution of retained teeth in edentulous subject

	Retained roots		Retained teeth		Total		
	n	%	n	%	N	n	%
Maxillae	8	7.27	5	4.55	65	13	11.81
Mandible	17	22.72	5	4.55	65	22	20

In 25 cases (71.42% of the total positive findings), the positive findings were on the level of bone surface, covered only by periosteum. In the remaining 10 cases (28.57% of the total positive findings) the positive findings were 4 mm under bone surface.

DISCUSSION

Scandrett et al. (1973) compared several methods of radiographic examination of the edentulous mouth. They examined a group of edentulous subjects by a Panorex radiogram, two by occlusal films, and 14 by periapical radiographs. The periapical survey revealed the largest number of residual pathoses, whereas the Panorex radiogram failed to confirm 25% and the occlusal films 56% of the same findings.

Periapical radiographs demonstrate good detail but their coverage is limited. Considerable time is involved in exposing, developing, and mounting films and, as radiographs are inserted in the mouth, the patients sometimes experience discomfort.

The reasons for the growing popularity of the panoramic radiography are:

— it provides a radiograph of the entire maxilla and mandible on a single film;

— it is timesaving;

— the film is not inserted into the mouth;

— the radiation dose to which the patient is exposed is smaller than when periapical films are used (Axelson, 1988).

In previous studies, the percentage of subjects with positive findings varied from 17% to 47%. To facilitate a comparison with the earlier studies, the results are presented in a table (Table 3).

Tab. 3 — Percentage of edentulous patients with retained roots in surveys with panoramic radiographs

Studies	N	Positive findings (%)
Prater 1968	224	16.5
Barclay and Donaldson, 1970	100	40.0
Mc Crorie, 1971	100	29.0
Perrelet et al, 1977	287	15.3
Keng et al, 1981	125	14.4
Keur, 1985	1135	14.2
Axselsson, 1988	250	11.1

* Results cit. from Axelson 1988

Compared with the previous studies, the presence of retained roots in the present study is on the same level (22.72%), but nearer to the lower threshold. Gradual decrease in the number of radiographic findings has been anticipated on account of the progress achieved in dentisty, increased use of radiographs before and after extraction, and a larger number of oral surgeons.

Our results did not corroborate the results of most studies (Axelson, 1988), which registered more root fragments in the maxilla. This may be due to a difference in the choice of sample. However, our results confirm those of Prater (1968) who, analyzing 1000 radiographs, diagnosed more retained roots in the mandible.

Ten of the edentulous patients (4.55%) had retained teeth. In previous reports (3, 4, 5), the percentages ranged between 1% and 8%.

Opinions differ as to the advisability of removing embedded root fragments and teeth. Some authors regard each retained root as a threat to the health of the patient and recommend for all retained fragments to be removed (Ennis, Berry, 1949; Mead, 1954). Others see no justification for a routine removal of root fragments which are embedded in the bone and reveal no clinical or radiographic signs of infection (Krstić et al., 1991).

Retained teeth which are completely impacted in the bone and which show no clinical symptoms or radiographic evidence of having developed enlarged follicular sacs or resorption are often left undisturbed (Axelsson, 1988).

Surgical intervention with unerupted teeth is not a routine procedure as bone destruction resulting from tooth removal is frequently extensive (Ennis, 1949).

Retained teeth located in the region of neuralgia, those showing radiographic evidence of an enlarged follicular sac or cyst formation, and superficially located teeth that have already or are likely to be exposed to the mouth should be removed (White, Weissman, 1977).

Our results for patients who were using a complete denture for five years or longer show that tooth roots, root fragments or teeth that were retained in the jaw had no influence on the health of the patient, local or general. Similar results were reported by Garcia et al. (1987) for a long-term study conducted on 33 edentulous patients. No change was noted over time in radiographic findings observed during the initial, or baseline, panoramic examination, and no new radiographic findings occurred over the 10-year study period.

CONCLUSIONS

The following conclusions were drawn on the basis of the obtained results.

1. Ortopantomographs provide a good picture of the situation that can be found in the edentulous mouth. Because of the easy application of film and low radiation exposure of the patient, it is recommend for use in preprosthetic preparation.

2. Retained roots and unerupted teeth, which bear no clinical symptoms or radiographic evidence of enlarged follicular sacs or resorption, are often left undisturbed.

3. Pathological findings could not be correlated with the sex of the patients.

4. Retained roots were frequently found in the mandible.

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РЕНДГЕНОГРАФСКИ НАЛАЗ КОД НОСИЛАЦА ТОТАЛНИХ ЗУБНИХ ПРОТЕЗА

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Резиме

Дијагностички добитак препротетског радиолошког испитивања је висок тако да је пре израде првих тоталних протеза потребно снимити вилице, чак и када нема клинички видљивих патолошких промена; међутим, ова врста истраживања још није постала практични стандард. Раније мишљење да сви ретинирани корени и њихови делови временом постају инфективни је превазиђено. То потврђују и наши налази, према којима се код 22,72% испитиваних могу рендгенолошки уочити заостали корени у безубим вилицама и поред петогодишњег ношења протеза. Налаз неизниклих зуба је позитиван код 4,55% од укупног броја пацијената (110 испитаника). Ни код једног пацијента нису регистровани клинички видљиве промене, а није било ни рендгенолошки видљивих знакова перифокалне инфламације. Може се закључити да ретиниране корене и неизникле зубе без рендгенолошки видљивог околног расветљења и клинички видљиве инфекције, уколико су потпуно прекривени коштаном структуром, није потребно уклањати из безубих уста.

Tab. 1 — Phytocoenological analysis of the community *Rusco-Querco-Carpinetum tilietosum tomentosae* subass. nova

The number of sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Date	9. 9. 1998					10. 9. 1998			2. 7. 1998				3. 7. 1998					5. 7. 1998				6. 7. 1998		6. 7. 1998				
Locality	Stražilovo								Elektrovojvodina									Venac				Matijevac		Zmajevac				
Altitude — m	350		320	300		330			450						480			450				450		450				
Exposition	N/NE		N	NE	N	NW			SW	N					NE	N		NNW	N	SE	N	N		N				
Slope (°)	2					3	2			10	2	5		2				5	10	5			10	2				
Canopy	0,7	0,6	0,8		0,7	0,5	0,4	0,8	09	0,8	0,7	0,9	0,8	0,9		0,8		0,9				0,8	0,7		0,9		
Average — dbh (cm)	40	35		30		30			40		30	35	50	30	40		30	25	30			40		30	50	35		
Average height (m)	20								18	30	18							20	25	18		20		25	12		20	
Size of the sampled area (m)	50x50	30x30							50x50									50x50										
Tree layer																												
Tilia tomentosa Moench	5.3	4.3	4.3	5.3	4.3	4.3	4.3	5.3	5.4	3.3	5.4	4.3	4.3	4.3	5.4	4.3	4.3	4.3	4.3	3.2	4.3	5.3	4.3	4.2	4.2	3.2	3.2	V
Carpinus betulus L.	2.1	2.2	2.2	+					1.1	4.3	1.1	3.3	+	1.1	1.1	2.2	3.3	4.3	3.2	3.2	3.3	1.1	2.1	3.1	2.1	4.2	1.1	V
Quercus petraea (Matt.) Liebl.		1.1	+	2.2	2.1	2.1	2.1	2.1	2.2	2.2	+			2.1	+			+	+	3.2	+	2.1	1.1	3.1	3.2	3.1	4.2	IV
Fagus moesiaca (Domin, Maty) Czeczott									1.1	+		1.1	r	+	2.1	1.1	+	1.1		2.2		1.1	2.1		1.1	2.1	2.1	III
Tilia cordata Moench			+							+				+				3.2	3.2			1.1	2.1	+	1.1	1.1	1.1	II
Acer platanoides L.							+	+			+		+				+	1.1	2.1		+	1.1				+	1.1	II
Primus avium L.											1.1							1.1	2.1	1.1	+	+				+		II
Ulmus glabra Huds.							+							+	+				1.1			+				1.1		II
Acer campestre L.						+					+								+							1.1		I
Sorbus torminalis (L.) Crantz							+																				+	I
Fraxinus ornus L.				1.1	+																							I
Acer pseudoplatanus L.																			2.1	+								I
Quercus cerris L.																										+	3.2	I
Shrub layer																												
Tilia tomentosa Moench	3.3	3.3	2.2	3.3	3.3	3.3	3.3	1.1	1.1	1.1	2.2	2.2	2.2	3.3	2.2	2.2	2.2	2.2	3.3	2.2	2.2	3.3	3.3	2.2	3.3	1.1	2.2	V
Acer platanoides L.	1.1	1.1	1.1	1.1		+			+			1.1	+		+							2.2	2.2	+	1.1	+	3.3	III

Acer campestre L.		1.1	1.1	1.1	2.2		1.1	1.1	1.1	+		1.1		+								2.2		2.2		1.1	1.1	III
Ulmus glabra Huds.	3.3	2.2		2.2			1.1		+			+		1.1	2.2				1.1				+	+	1.1	1.1	1.1	III
Staphylea pinnata L.	2.2	2.2	2.2	3.3	2.2			1.1		+									1.1	+		+		1.1	3.3	2.2	1.1	III
Carpinus betulus L.		2.2	2.2							+			1.1			2.2		2.2	2.2	2.2	+	3.3		2.2	1.1	1.1	2.2	III
Cornus mas L.					1.1	2.2	2.2	2.2	+					+								+	+	3.3	2.3	1.1	2.2	III
Sambucus nigra L.						+	1.1		+	+			+			+	+		2.2	2.2					+	1.1	+	III
Crataegus monogyna Jacq.		1.1		1.1		1.1	1.1	1.1						+	+					1.1				2.2	1.1	2.1		II
Fagus moesiaca (Domin, Maty) Czeczott									+			+			+		+	+				1.1	1.1		1.1	2.1	1.1	II
Evonymus europaeus L.	1.1	1.1	1.1							+		+								2.1				+	1.1	2.1		II
Prunus avium L.					1.1							+						1.1	2.2	1.1	+	+				1.1	1.1	II
Cornus sanguinea L.	1.1	2.2	1.1	2.2	1.1			2.2																+				II
Viburnum lantana L.	2.2	2.2		+	1.1	+													+									II
Ligustrum vulgare L.	1.1		1.1	1.1				2.2													+			+				II
Tilia cordata Mill.			1.1							+								1.1	2.2			1.1		+				II
Corylus avellana L.	1.1		1.1	1.1								+												+				I
Fraxinus ornus L.				1.1			+	1.1																			+	I
Ruscus aculeatus L.		2.2		2.2	2.2			2.2																				I
Rosa arvensis Huds.																							+	+			+	I
Sorbus torminalis (L.) Crantz								1.1																+				I
Robinia pseudoacacia L.						2.1	1.1																					I
Rubus hirtus W. et K.										1.1				1.1														I
Acer pseudoplatanus L.																			2.1									I
Evonymus verrucosus L.				1.1																								I
Herb layer																												
Stellaria holostea L.	2.2	2.2			2.2	2.2	2.2		1.1	+	1.1	2.2	+	1.1	3.3	1.1	2.2	2.2	3.3		3.3	2.2	2.2	3.3	1.1	2.2	1.1	V
Galium odoratum (L.) Scop.	1.1	2.2	2.2					1.1	1.1	1.1	1.1	+	+	1.1	1.1	1.1	+	3.3	1.2		1.1	2.2	2.2	1.1	+	2.2	1.1	IV
Hedera helix L.	2.2	2.2	2.2		2.2	2.2		2.2	2.2	1.1	1.1	2.2	1.1	1.1	1.1		2.2	3.3	2.2	3.4	1.1	2.2	2.2			2.2	2.2	IV
Glecboma hirsuta W. et K.					1.1	1.1	2.2	2.2	1.1	1.1	1.1	1.1		1.1	+	1.1	1.1	3.3	3.4	4.4	2.2	1.1	2.2	1.1	1.1	1.1	2.2	IV
Rubus hirtus W. et K.	3.3	1.1	2.2						4.4	5.5		4.4	3.3	4.4	5.5	2.2	1.1	3.3	4.4	2.2	3.3	4.4	4.4	4.4	3.3	3.3	4.4	IV
Geum urbanum L.					1.1	1.1	1.1		+		1.1		1.1	1.1	1.1	1.1	1.1	2.1	1.1	1.1	1.1	+	+	+	+	1.1	1.1	IV

Euphorbia amygdaloides L.		1.1			1.1	2.1	2.2	1.1	+			+	+		+		+	+	1.1	1.1		+	+	1.1	+	+	1.1	IV
Helleborus odorus W. et K.	1.1	1.1	1.1			1.1	1.1	1.1	+		+	+		+	+	+	1.1	1.1			+	+		+		+		IV
Pulmonana officinalis L.	2.2	2.2	2.2		1.1	1.1	1.1		1.1	+	+		+	1.1		1.1			1.2							+	+	+	III
Festuca drymeia Mert. et Koch		1.1			3.3	1.1		2.2	1.1	+		1.1	+	+	+	+	1.1	+						1.1	+			III
Alliaria officinalis Andrz.							1.1		+	+	+	+				+		3.3	1.1	1.1		+	+	+		+	+	III
Galeobdolon luteum Hudson	4.4	4.4	4.4							3.3	+		+	+		+		3.3	3.3	4.4		1.1	1.1					III
Melica uniflora Retz.					2.2	2.2	2.2		+		2.2	1.1		2.2		1.1	1.1			1.1		1.1		2.2		2.2		III
Geranium robertianum L.						2.2	2.2		+		1.1	1.1				+		1.1	1.1	2.3	+	+	+			1.1		III
Asperula taurina L.	1.1	2.2					2.2	2.2	1.1		1.1		+			1.1	1.1		1.1		1.2					1.1		III
Acer campestre L.								1.1	1.1			+		+	+	+	+	+		1.1		1.1	+				+	III
Mercurialis perennis L.	2.2						1.1	1.1		+	+		+			+	1.1		1.1	1.1				+		1.1		III
Asarum europaeum L.	1.1	2.2			1.2	1.1	1.1	2.2		1.1		+	+					1.2	1.1		1.1							III
Dactylis glomerata L.					1.1				1.1		+		+	+	+	2.2						1.2			+		2.2	II
Rumex sanguineus L.									+		+		1.1		+	+	+							+	+	+	1.1	II
Brachypodium silvaticum (Huds.) P. Beauv.	2.2		1.1		1.1	2.2	2.2																	+	+	1.1	+	II
Ruscus aculeatus L.	2.2	3.3	3.3		4.3	3.3	3.3	3.3													3.3			1.2				II
Polygonatum multiflorum (L.) All.	1.1										+			+		+			+			+		+		+		II
Ruscus hypoglossum L.			1.1		1.1					2.1		+	+					3.3				1.2	2.2		+			II
Campanula trachelium L.									+				+	+	+		+	1.1	1.1		+						+	II
Quercus petraea agg.							2.2		1.1					+								2.1	2.1	1.1		+	1.1	II
Mycelis muralis (L.) Rchb.			1.1															+				+	+	1.1		+	+	II
Geranium phaeum L.											+		+	+		1.1	+				+					3.3		II
Sanicula europaea L.													1.1	1.1	+	+						+		+	+			II
Lilium martagon L.																	+	1.1		1.1	+	+				1.1	1.1		II
Evonymus europaeus L.			1.1			1.1					+	+								2.1				+				II
Anthriscus silvestris (L.) Hoffm.							1.1					+				+			1.1	1.1				+				II
Rosa arvensis Huds.							2.2		+							+		1.1						+	+			II
Lamium maculatum L.							1.1		+		+	+				+									+			II
Galium silvaticum L.								1.1							+					1.1		+			1.1		+	II
Heracleum sphondylium L.									+	+				+				1.1				1.1			+			II

Lapsana communis L.									+				+			+				+		+					+	II
Fragaria vesca L.									+				+			+	+		1.1						+			II
Arum maculatum L.									+	1.1	+										+	+				+		II
Prunus avium L.																+		2.2	1.1	1.1	+	+						II
Ligustrum vulgare L.		1.1			1.1			1.1										1.1			+							I
Acer platanoides L.			1.1						+										1.1						+	+		I
Viola silvestris Lam.						1.1	1.1		+											+					+			I
Aegopodium podagraria L.											+		+	+											2.3	2.3		I
Lathyrus vernus (L.) Bernh.					1.1										+					+					+			I
Carex pilosa Scop.														1.1	+									3.4		2.2		I
Carpinus betulus L.	1.1								+															1.1				I
Tilia tomentosa Moench			1.1								+							1.1										I
Crataegus monogyna Jacq.							1.1										+							+				I
Stachys silvatica L.										+		+		+														I
Chaerophyllum temulum L.										+		1.1		+														I
Galeopsis speciosa Mill.														1.1	+					1.1								I
Cornus mas L.																+								1.1	1.1			I
Carex silvatica Hudson	2.2				+								+															I
Viola hirta L.					1.1								+										+					I
Lonicera caprifolium L.					1.1																				+			I
Galium cruciatum (L.) Scop.						1.1		2.2																				I
Lithospermum purpurocoeruleum L.						1.1	1.1																					I
Aremonia agrimonioides (L.) Neck						1.1			+																			I
Urtica dioica L.							1.1					+																I
Prunella vulgaris 1.									+				1.1															I
Poa nemoralis L.													+														+	I
Aconitum vulparia Reichenb.														+							+							I
Platanthera bifolia (L.) Rich.					+																							I
Viburnum lantana L.					1.1																							I
Fraxinus ornus L.					1.1																							I
Hypericum perforatum L.						1.1																						I

Chelidonium majus L.						1.1																					I
Rubus tomentosus Borkh.									1.1																		I
Arctium lappa L.									+																		I
Stellaria media (L.) Mill.										+																	I
Acer pseudoplatanus L.																		1.1									I
Sambucus nigra L.										+																	I
Scrophularia nodosa L.												+															I
Veronica serpyllifolia L.												+															I
Cardamine bulbifera (L.) Crantz												+															I
Galium pseudoaristatum Schur												+															I
Veronica chamaedrys L.												+															I
Campanula persicifolia L.												+															I
Cardamine enneaphyllos (L.) Cr.														+													I
Ulmus glabra Huds.																		1.1									I
Tamus communis L.																									+		I
Convolvulus arvensis L.																										+	I
Astragalus glycyphyllos L.																											+	I
Quercus cerris L.																											+	I

*28 Degree of presence