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Biochemistry

ANTIRADICAL EFFICIENCY OF SOME STANDARD PHENOLIC COMPOUNDS

SIMONISHVILI SH., SHALASHVILI A., ZAMBAKHIDZE N., TARGAMADZE I., GOGAVA M., MITAISHVILI T., CHRIKISHVILI D., UGREKHELIDZE D.

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Abstract

Antiradical efficiency of 31 standard phenolic compounds, including simple phenols, hydroxybenzoic acids, aldehydes, hydroxy-cinnamic acids and coumarins has been studied by means of a stable free radical, 1,1-diphenyl-2-picryl hydrazyl (DPPH$^{\bullet}$). It was shown that phenolic compounds are potential binding agents of free radicals, and their activity with respect to DPPH$^{\bullet}$ is defined by chemical structure of their molecules. Among phenolic compounds, esculetin, pyrogallolcarboxylic acid, gallic acid, caffeic acid, protocatechuic acid, pyrogallol and protocatechuic aldehyde have high antiradical efficiency.

Key words: 1,1-diphenyl-2-picryl hydrazyl, free radicals, phenolic compounds, interrelation of structure and activity.

Introduction

Continuing study of antioxidant properties of phenolic compounds, [Shalashvili et. al. 2006; 2002], we investigated antiradical efficiency of some standard phenolic compounds (simple phenols, hydroxybenzoic acids, aldehydes, hydroxy-cinnamic acids and coumarins) by means of a stable free radical, 1,1-diphenyl-2-picryl hydrazyl (DPPH$^{\bullet}$) [Sanchez-Moreno et al., 1998; Sanchez-Moreno, 2002].

Materials and Methods

31 standard phenolic compounds have been investigated. Including simple phenols: pyrocatechin (1,2-dihydroxybenzene), resorcinol (1,3-dihydroxybenzene), hydroquinone (1,4-dihydroxybenzene), pyrogallol (1,2,3-trihydroxybenzene), phloroglucinol (1,3,5-trihydroxybenzene) ("Reachim", Russia), and orcinol (5-methylresorcinol) ("Austramal-Preparate", Austria); hydroxybenzoic acids: salicylic acid (o-hydroxybenzoic acid), m-hydroxybenzoic acid, p-hydroxybenzoic acid, β-resorcylic acid (2,4-dihydroxybenzoic acid), gentisic acid (2,5-dihydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), methylgallic acid (4,5-dihydroxy-3-methoxybenzoic acid), syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid) ("Reachim", Russia), γ-resorcylic acid (2,6-dihydroxybenzoic acid) ('Theodor Schuchardt", Munich), and pyrogallolcarboxylic acid (2,3,4-trihydroxybenzoic acid)

("Chemapol", Prague); hydroxy-cinnamic acids: cinnamic acid, ferulic acid (4-hydroxy-3-methoxycinnamic acid), and sinapic acid (4-hydroxy-3,5-dimethoxycinnapic acid); coumarins: coumarin, umbelliferone (7-hydroxycoumarin), scopoletin (7-hydroxy-6-methoxycoumarin), esculetin (6,7-dihydroxycoumarin) ("Chemapol", Prague), esculin (6,7-dihydroxycoumarin-6-glucoside; esculetin-6-glucoside) ("Merk", Germany); protocatechuic aldehyde (3,4-dihydroxybenzaldehyde) ("Reachim", Russia), and vanillin (4-hydroxy-3-methoxybenzaldehyde) and α-tocopherol ("Sigma", USA).

40μM of 1,1-diphenyl-2-picryl hydrazyl ($DPPH^{\bullet}$) solution in ethanol has been prepared. To this solution 3 ml of a solution of investigated phenol of 5 various concentrations was added. After agitating, samples were incubated for 5 minutes at room temperature. After incubation, optical density of a solution was determined on a spectrophotometer "СФ-26" (Russia) [Sanchez-Moreno et al., 1998; Pochinok et al., 1985]. Each variant of this experiment was repeated 5 times. The obtained data were processed statistically by the computer program "MS Excell". Antiradical efficiency (AE) has been calculated by the formula: $AE = 1 / EC_{50} \bullet T_{EC50}$, where EC_{50} expresses that amount of antioxidant, which reduces initial optical density of $DPPH^{\bullet}$ · ethanolic solution by 50%. For each compound this quantity is evaluated by means of the calibration curve and expressed as quantity of an antioxidant in grams per kg of $DPPH^{\bullet}$. T_{EC50} is an interval of time during which in the incubating medium there is a reduction of $DPPH^{\bullet}$ concentration by a half.

Results and Discussion

According to the data of the Table 1, from investigated phenolic compounds by their antiradical efficiency esculetin, pyrogallol carboxylic acid, gallic acid, caffeic acid, protocatechuic acid, pyrogallol and the protocatechuic aldehyde are distinguished, which efficiencies several times exceed antiradical efficiency of α-tocopherol.

From the studied 6 simple phenols, high antiradical efficiency was displayed by pyrogallol (AE = 1.47). Pyrocatechol (AE = 0.62) and hydroquinone (AE = 0.59) are rather less active, and resorcinol, phloroglucinol and orcin do not possess antiradical efficiency. Apparently, in case of simple phenols, for display of high antiradical efficiency, presence of three consistently located hydroxyl groups in a molecule is necessary, as it is observed in a pyrogallol molecule.

From hydroxybenzoic acids, the mono-hydroxybenzoic acids: o-, m- and p-hydroxybenzoic acids have no antiradical efficiency; antiradical efficiency of dihydroxy- and trihydroxy benzoic acids depends on a position of hydroxyl groups in an aromatic ring. In protocatechuic acid, presence of hydroxylic groups in meta-and para-positions concerning a carboxyl group causes antiradical efficiency of this acid (AE = 1.51). Replacement in this molecule of a hydroxyl group by methoxy group causes decrease in antiradical efficiency almost five times (vanillic acid, AE = 0.31), and in case of protocatechuic aldehyde (AE = 1.42), replacement in a position 3 of methyl group completely suppresses its antiradical efficiency (vanillin, AE = 0). Antiradical efficiency of gentisic acid makes 0.43, while β- and γ- resorcylic acids do not possess antiradical efficiency. According to literary data [Rice-Evans et al., 1996], α-resorcylic acid displays high antioxidant activity (TEAC = 2.15 mM) which is equivalent to activity of Trolox. (TEAC = Trolox Equivalent Antioxidant Capacity).

From trioxybenzoic acids, pyrogallolcarboxylic acid and gallic acid possess ample antiradical efficiency (AE = 1.92 and AE = 1.88, accordingly), that can be caused, as well as in case of pyrogallol (AE = 1.47), by presence in a cycle of three subsequent hydroxyl groups. It is necessary to notice, that in a gallic acid molecule replacement of hydroxyl group by methoxy group in a position3, suppresses antiradical efficiency 2.8 times (methylgallic acid, AE = 0.66), and

replacement of two methoxy groups in positions 3 and 5, decreases the value of AE 4.7 times (syringic acid, AE = 0.40).

From hydroxy-cinnamic acids, the highest antiradical efficiency was displayed by caffeic acid (AE = 1.58). Replacement of a hydroxyl group of this acid by methoxy group in a position 3, as well as in case of protocatechuic acid, reduces antiradical efficiency 4.2 times (ferulic acid, AE = 0.37). As a result of study of equivalent antioxidant activity of Trolox, it has been appeared that replacement of a hydroxyl group with methoxy group in a position 3 of coffee acid (TEAC - 1.2 mM) considerably increases antiradical efficiency (TEAC = 1.9 mM) [Rice-Evans et al., 1996].

It should be also noticd that in comparison with ferulic acid, sinapic acid has displayed twice high antiradical efficiency (AE = 0.77). According to Rice-Evans et al. (1996), placement of ethylenic group between a cycle of phenol and a carboxyl group, as it takes place in p-coumaric acid, renders strong effect on the reductive properties of a hydroxyl group (TEAC = 2.2 mM) in comparison with cinnamylic acid (TEAC = 0), and values of TEAC for o-coumaric and p-coumaric acids are 1.21 and 0.99, correspondingly. According to our data, cinnamyic acid, o- coumaric and p- coumaric acids do not possess antiradical efficiency.

From the investigated 5 coumarins (coumarin, umbelliferone, esculetin, esculin and scopoletin) only esculetin displayed high antiradical efficiency (AE = 2.17). Apparently, in this case antiradical activity is caused by presence of hydroxyl groups in an ortho- and meta- positions.

Investigated phenolic compounds, by duration of influence, belong to responsive rapidly reacting compounds as their T_{EC50} amounts to 1.3 - 5 minutes (Table) [Sanchez-Moreno et al, 1996].

Table 1. Antiradical efficiency of some standard phenolic compounds

Standard phenolic compound	**EC_{50} (g of antioxidant per kg of $DPPH^{\cdot}$)**	**T_{EC50} (minutes)**	**AE ($*10^3$) (antiradical efficiency)**
Simple phenols:			
Hydroquinone	340±2.19	5	0.59
Pyrocatechin	319±5	5	0.62
Pyrogallol	170±5.25	4	1.47
Hydroxybenzoic acids:			
Gentisic acid	468±5.83	5	0.43
Protocatechuic acid	132±5.83	5	1.51
Vanillic acid	638±1.61	5	0.31
Pyrogallolcarboxylic acid	103±5.35	5	1.92
Gallic acid	106±5.50	5	1.88
Methylgallic acid	425±8.19	3.5	0.66
Syringic acid	510±3.21	5	0.40
Aldehydes:			
Protocatechuic aldehyde	140±4.16	5	1.42
Hydroxycinnamic acids:			
Caffeic acid	127±3.85	5	1.58
Ferulic acid	553±8.70	5	0.37
Sinapic acid	638±4.72	2	0.77
Coumarins:			
Esculetin	353±6.54	1.3	2.17
α-Tocopherol	625±6.02	5	0.32

In plants among simple phenols hydroquinone and its derivatives occur which are found in plants of families *Ericaceae, Rosaceae, Proteaceae and Compositae.* Pyrocatechol is found in

bulbs of onions (*Allium cepa*) and in grapefruit fruits. Pyrogallol is discovered cones of sequoia, and also in onion (*Allium cepa*). From hydroxybenzoic acids in plants protocatechuic acid and vanillic acid are widespread, gentisic and gallic acids are met often enough, and syringic acid is rather rare. In many berry plants (the wild strawberry, bilberry, currant, gooseberry) glucosides of protocatechuic and gallic acids are found out. Free gallic acid is detected in tea leaves. From hydroxy-cinnamic acids, coffee, ferulic and sinapic acids are widespread in plants, and plants of families *Umbelliferae, Rutaceae, Solanaceae, and Leguminosae* basically contain coumarins. Scopoletin is isolated from pericarp of a horse-chestnut [Zaprometov, 1993; Harborne, Williams, 1969].

Thus, from the investigated 31 phenolic compounds, 14 possess antiradical efficiency and the majority of them are widespread. They are consumed by humans with food rendering beneficial effect on their health [Pokorny et al. (eds) 2001].

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BIOREMEDIATION OF OIL-CONTAMINATED SOILS BY APPLYING OF MICROSCOPIC FUNGI

KHOKHASHVILI I., KUTATELADZE L., ALEKSIDZE T., KHAREBASHVILI M., KVESITADZE E., LASKHISHVILI M.

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(Received October 15, 2008)

Abstract

Oil-destructing strains of microscopic fungi were reveled from oil-polluted territories of Georgia and from the collection of Durmishidze Institute of Biochemistry and Biotechnology. Among the tested 130 strains 10 strains with high oil-destructing abilities were selected. Conditions of cultivation of high active oil-destructor strains have been established. In the case of application of destructor strains - *Chaetomium* sp. J 2-2 and *Trichoderma viride* J3-1 under laboratory and field conditions the extent of oil destruction in oil-polluted black and red soils made 85-90%. This indicats the high destructive activity of the strains and makes possible their application in bioremidation technologies of oil-polluted soils. According to high bio-degrading activity of the studied fungi the technological scheme of bioremediation of the polluted soils and obtaining of bio-preparations was elaborated.

Key words: biodegradation, microscopic fungi, oil-destructor strains, bio-preparation

Introduction

Cleaning of environment from various toxic contaminants, including oil spill and oil products is one of the acute problems in the world [Wagner et al., 1964]. At present, detoxification ability of microorganisms is being studied in many countries all over the world. Bacteria and basidial or white rot fungi capable to assimilate oil are well studied [Kvesitadze G., Kvesitadze E., 2000]. Detoxification ability of microscopic fungi is lees investigated. However, according to the recent data, microscopic fungi and yeasts also possess the mentioned property [Eaton, Hale, 1993; Kholodenko et al., 2001].

Materials and Methods

Objects of investigation were microscopic fungi cultures isolated from oil-contaminated soils and collection strains of microscopic fungi of different genera held in Durmishidze Institute of Biotechnology and Biochemistry.

The oil-utilization capability of microscopic fungi were estimated by conidial grow on solid medium according to three point system (+ - poor growth, ++ - good growth, +++ - intensive growth). The biodegradation level in liquid medium was estimated by quantity of residual oil.

Results and Discussion

In order to select strains, active destructors of oil products, the screening of freshly isolated cultures and collection strains of microscopic fungi held in the Institute, were carried out on oil-containing nutrient media.

It was found that 44 cultures out of 130 test ones grew well at low concentrations of oil products (10 mg/l), 19 cultures – at 20 g/l; 10 cultures grew well at high concentrations (30 mg/l) and only 1 culture displayed moderate growth at the concentration of 40 mg/l.

For the next investigations, cultures growing well on solid nutrient medium, containing 30 mg/l of oil were chosen. These cultures are as followed: *Chaetomium* sp.J2-2, *Chaetomium* sp. J3-3, *Trichoderma lignorium* M 3-3, *Trichoderma viride* J3-1, *Gymnoascus* sp. J2-1, *Aspergillus versicolor* J1-1, *Aspergillus* sp.J 1-2, *Aspergillus terreus* J2-3, *Aspergillus niger* J3-4; *Trichoderma viride* J 3-5. For quantitative estimation of assimilated oil products, microscopic fungi were grown in modified Czapek's liquid medium. Biodegradation ability of microscopic fungi was estimated by the amount of residual oil in culture liquid. Three strains of microscopic fungi – *Chaetomium* sp. J2-2; *Trichoderma viride* J 3-1; *Aspergillus versicolor* J1-1 utilized almost 100% of oil (Fig.1. and Fig. 2).

It should be mentioned that these strains were isolated from oil-contaminated soils and their relatively high degradation ability might be explained by adaptation of oil containing environment.

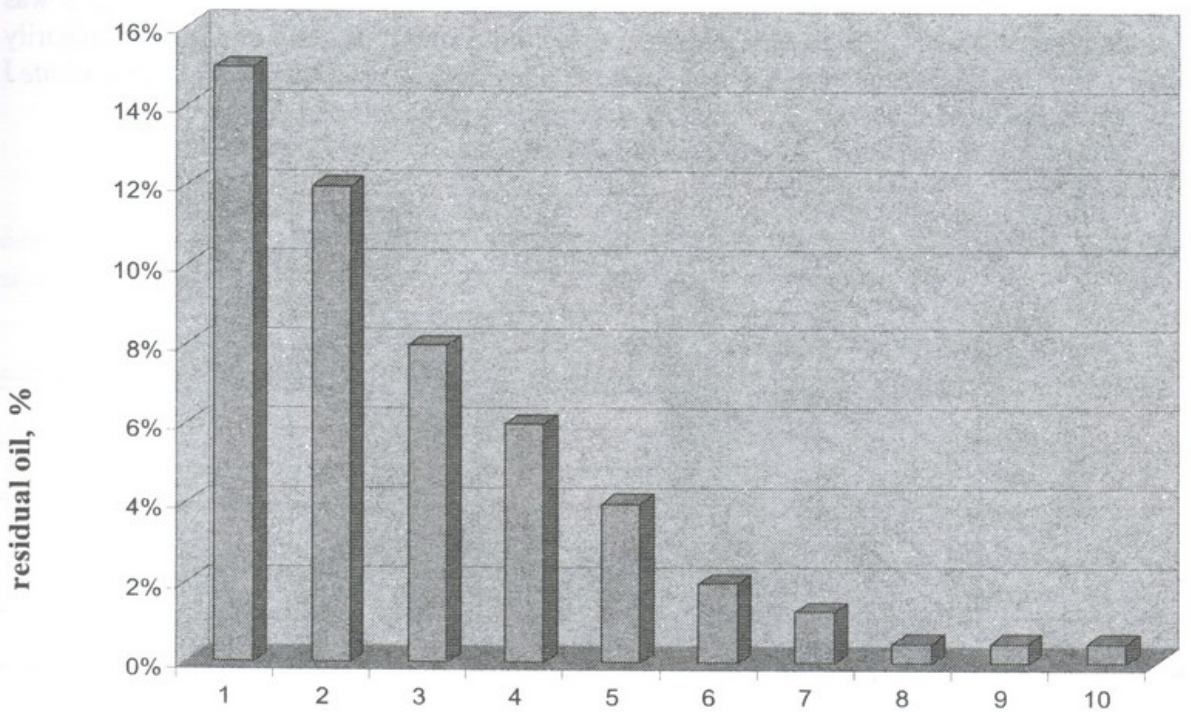

Fig. 1. The degradation of the oil by the microscopic fungi; the cultivation condition: 30–40^{0}C, pH 4.5—8.5, 72-96 h. The oil concentration in nutrient medium - 30g/l.
1 - Aspergillus Níger J3-4; 2 - Aspergillus venti J1-2; 3 - Trichoderma viride J3-5; 4 - Chaetomium sp. J3-3; 5 - Aspergillus terreus J2-3; 6 - Gymnoaseus sp. J2-1; 7 - Trichoderma lignorium M3-3; 8 - Aspergillus versicolor J1-1; 9 - Trichoderma viride J3-1; 10 - Chaetomium sp. J2-2.

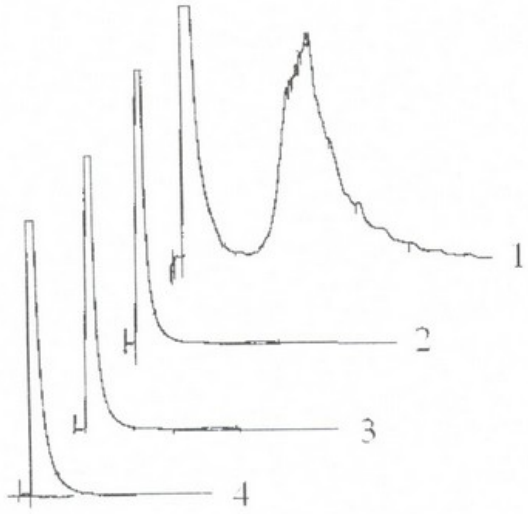

Fig. 2. Chromatograhphy of total hydrocarbons of residual oil.
1 - The control; 2 - *Trichoderma viridae* J1-1;
3 - Aspergillus versicolor J1-1; 4 - Chaetomium sp. J2-2

Bioremediation ability of strains, selected for applying in remediation technologies was studied under laboratory, sterile and natural modeling conditions, on soils preliminarily contaminated with oil. The results for two test strains – *Chaetomium* sp. J2-2, *Trichoderma viride* J 3-1 are shown in Fig.3 and Fig.4.

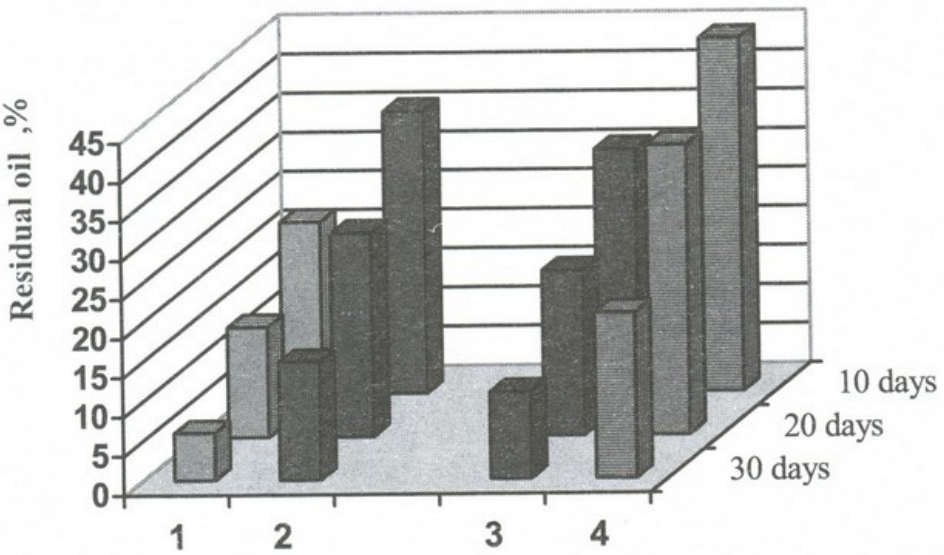

Fig. 3. The degradation of the oil by the microscopic fungi in sterile conditions.
The cultivation condition: 30–40^{0}C, pH 5.5-6.0. The oil concentration - 30g/kg.
1 - black soil+oil+*Chaetomium* sp.J2-2; **2** - red soil+oil+*Chaetomium* sp. J2-2;
3 - black soil+oil+*Trichoderma viride* J3-1; **4** - red soil+oil+*Trichoderma viride* J 3-1;

As it seen from Fig.3, biodegradation level of oil in local microorganisms from contaminated soils equals to 40-50%, and in case of additional introduction of strain-destructors, it may reach 90%. From the same data, it may be concluded that 50% of oil from the soil is utilized in 10-14 days subsequent to introduction of biomass; that of 85% – in 30 days, and almost complete

cleaning is reached in 1.5-2 months in case of contamination with low and moderate concentrations and in 2.5-3 months – with high concentrations.

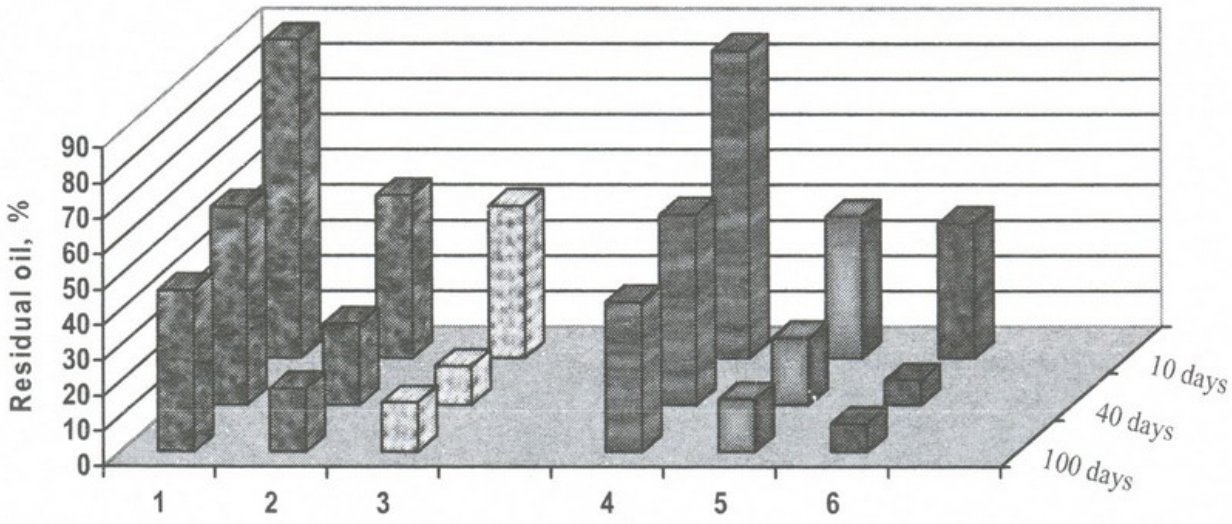

Fig. 4. The degradation of the oil by the microscopic fungi in natural modeling conditions. The cultivation condition: 30–40^{0}C, pH 5.5-6.0. The oil concentration - 30g/kg.
1 - red soil+oil; **2** - red soil+oil+*Trichoderma viride* J3-5; **3** - red soil+oil +*Chaetomium* sp. J2-2; **4** - black soil+oil; **5** - Black soil+oil+*Trichoderma viride*J3-1; **6** - black soil+oil + *Chaetomium* sp. J2-2.

By applying of mathematical modeling, the rate of biopreparation application subjected to contamination level was established for cleaning of contaminated soils. The obtained results are shown in Table 1.

Table 1. The rate of biopreparation application subjected to contamination level. S (%) - contamination level; D (kg) – the rate of preparation application in terms of 1 ton of contaminated soil.

S (%)	1	2	3	4	5	6	7	8	9	10
D (kg)	0,02	0,04	0,08	0,11	0,15	0,19	0,24	0,29	0,34	0,4
S (%)	11	12	13	14	15	16	17	18	19	20
D (kg)	0,46	0,54	0,65	0,77	0,9	0,99	1,11	1,26	1,43	1,6
S (%)	21	22	23	24	25	26	27	28	29	30
D (kg)	1,72	1,85	1,98	2,11	2,25	2,39	2,54	2,69	2,84	3,0
S (%)	31	32	33	34	35	36	37	38	39	40
D (kg)	3,16	3,33	3,5	3,67	3,85	4,03	4,22	4,41	4,6	4,8
S (%)	41	42	43	44	45	46	47	48	49	50
D (kg)	5,0	5,04	5,16	5,28	5,85	5,98	6,11	6,24	6,37	7,0
S (%)	51	52	53	54	55	56	57	58	59	60
D (kg)	7,14	7,28	7,42	7,56	8,25	8,4	8,55	8,7	8,85	9,6

It was also determined that multiplicity of soil treatment depends on contamination level S (%) (Table 2).

Table 2. Multiplicity of soil treatment depends on contamination level S (%))

S (1 – 7)	S (8 – 14)	S (15 – 24)	S (25 – 34)	S (35 – 60)
1 treatment	2 treatment	3 treatment	4 treatment	5 treatment

Soil bioremediation efficiency was estimated by germinating intensity of wheat grown on it. The initial (clean) plot, that of contaminated with the same oil concentration and cleaned spot by biopreparation were tested. Wheat was sown simultaneously on all three plots. The growth intensity of seeds on the plot treated by biopreparation was almost the same as that of the clean soil. The obtained results are shown in Fig. 5.

a) **b)**

Fig. 5. The estimation of soil bioremediation efficiency by germinating intensity of wheat.
a) Oil-contaminated soil. b) Soil cleaned by biopreparation

It should be mentioned that the biopreparations obtained from selected cultures are nontoxic and nonpathogenic; in addition, they are not explosive and inflammable. Thus, their application is ecologically harmless.

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ნავთობით დაბინძურებული ნიადაგების ბიორემედიაცია მიკროსკოპული სოკოების გამოყენებით

ხოხაშვილი ი., ქუთათელაძე ლ., ალექსიძე თ., ხარებაშვილი მ., კვესიტაძე ე., ლასხიშვილი მ.

დურმიშიძის ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტი

(მიღებულია 15.10.2008)

რეზიუმე

საქართველოს ნავთობპროდუქტებით დაბინძურებული ტერიტორიებიდან გამოყოფილ და დურმიშიძის ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტში არსებული მიკროსკოპული სოკოების კოლექციის შტამებიდან სკრინინგის შედეგად გამოვლენილია ნავთობის დესტრუქტორი შტამები. 130 გამოკვლეული შტამიდან შერჩეულია მაღალი დეგრადაციის უნარის მქონე მიკროსკოპული სოკოს 10 შტამი. დადგენილია ნავთობის მაღალაქტიური დესტრუქტორი შტამების კულტივირების პირობები. ლაბორატორიულ და საველე ბუნებრივ პირობებში ნავთობით დაბინძურებულ შავმიწა და წითელმიწა ნიადაგებში შტამ-დესტრუქტორების – *Chaetomium* sp. J 2-2 და *Trichoderma viride* J3-1-ის შეტანის შემთხვევაში ნავთობის დეგრადაციის ხარისხი შეადგენს 85–90%-ს, რაც მიუთითებს შტამების მაღალ დესტრუქციულ აქტივობაზე და იძლევა მათი გამოყენების შესაძლებლობას ნავთობით დაბინძურებული ნიადაგების ბიორემედიაციის ტექნოლოგიებში. შერჩეული მიკროსკოპული სოკოების მაღალ ბიოდეგრადაციულ აქტივობაზე დაყრდნობით დამუშავებულია ბიოპრეპარატის მიღებისა და დაბინძურებული ნიადაგების ბიორემედიაციის ტექნოლოგიური სქემა.

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QUALITATIVE DETECTION OF GENETICALLY MODIFIED ORGANISMS

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Abstract

Genetically modified organisms (GMO) screening assays were developed using dried powders as certified reference materials containing 0-5% Roundup Ready soybean and maize Bt-176 as well as seeds of 100% Roundup Ready Soya. Genomic DNAs were extracted by Qiagen DNeasy plant mini kit. Amplification quality of DNAs was assessed by plant specific polymerase chain reaction (PCR) appropriate for detection of chloroplast genome conserved sequences. The PCRs corresponding to 35S promoter and the NOS terminator revealed the presence of transgenic material in all GMO containing certified reference materials. No amplification signal was exhibited in negative water and non-GM samples. The high sensitivity of 0.1% GMO was achieved for screening methods. The results obtained show that DNA-based assays, described in this study, permit reliable, sensitive and rapid qualitative detection of genetically modified organisms.

Keywords: genetically modified organisms (GMOs), screening of GMOs, 35S promoter, NOS terminator, DNA analysis.

Introduction

Worldwide distribution of genetically modified organisms (GMOs) has generated particular interest in these organisms in each country. During last decade the share of GMOs and products derived thereof increased exponentially in the global food production and trade [James, 2006]. In many countries, monitoring of genetically modified food products is regulated by legislation. In the EU, food products containing GMO material above the threshold of 0.9% need to be labeled [European Commission, Regulations, 2003a, 2003b]. Implementation of the regulatory laws needs reliable GMO detection methods. DNA-based polymerase chain reaction is recognized as the most useful technique for analysis of genetically modified organisms [Anklam et al., 2002]. Qualitative detection aimed at screening purposes is the first important step in GMO analysis. Several PCR systems corresponding to GMO regulatory elements, such as 35S promoter from Cauliflower Mosaic virus and the NOS terminator from *Agrobacterium tumefaciens*, have been verified for screening transgenic products [Pietsch et al., 1997; Studer et al., 1997; Lipp et al., 1999]. However, screening methods have problem with detection sensitivity, generally it is lower than the detection limit required by legislation. The aim of this study was to develop and optimize GMO screening methods relevant to EU legislation.

Materials and Methods

Plant material. Roundup Ready soya and Bt-176 maize were used as GM plants. Certified reference material (ERM-BF-410) of GM Soya bean powder set containing 0-5% Roundup Ready and maize GMO Standard (ERM-BF-411) set for 0-5% Bt-176 were purchased commercially from Fluka. The seeds of 100% Roundup Ready Soya was provided from European Institution.

Genomic DNA extraction. The soybean seeds were ground by mixer to obtain a fine powder. The certified reference materials were in dried powdered form. DNeasy plant mini kit (Qiagen) was chosen as the useful method for DNA isolation based on the results of our previous study [Kutateladze, et al., 2005]. Genomic DNAs were purified from 100 mg of powdered samples. The DNA quality and amount was assessed by agarose gel electrophoresis.

Oligonucleotide Primers. The plant-specific and GMO-specific PCR primer sequences were selected based on the published data [Pietsch et al., 1997; Studer et al., 1997]. The primers were synthesized and purified by MWG Biotech.

PCR Conditions. Polymerase chain reactions were performed with a thermal cycler Techne TC-412. The PCR reaction was carried out in final volume of 25 µl using 0.8 U GoTaq™ DNA polymerase with 1x green GoTaq™ reaction Buffer (pH 8.5) (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Promega), 0.4 µM of each primer, and 1µl (50-80 ng) of genomic DNA.

The PCR cycling profile for primers plant1/plant2 was: Initial denaturation 1 cycle at 95°C for 4 min, followed by 35 cycles of 95°C for 30s, 55°C for 30s, 72°C for 2 min; final extension 72^0C 5 min. The identical PCR conditions were applied for the two pairs of GMO-specific primers 35S1/35S2 and NOS1/NOS2 that were as follows: denaturation for 10 min at 95°C, amplification for 20s at 95°C, for 40s at 54°C, for 40s at 72°C; number of cycles 35 cycles; final extension for 5 min at 72^0C.

Agarose Gel Electrophoresis. The both genomic DNAs and PCR products were analyzed using agarose gel electrophoresis. The gel was prepared with 1.0 and 2.0% of agarose (Promega) for genomic and amplified DNA, respectively, in Tris Borate EDTA (TBE) 1x buffer with 1µg/ml of Ethidium Bromide (EtBr). After electrophoresis the DNA bends were visualized and photographed by Digital still camera (DSC-S600, Sony).

Results and Discussion

Qualitative detection procedure, described in this article, consists of several steps: genomic DNA extraction and evaluation; plant-specific PCR and GMO screening based on the detection of transgenic regulatory elements.

Genomic DNAs were extracted from each sample together with non-template (water) control. Assessment of the DNAs by agarose gel electrophoresis exhibited high purity and integrity of the samples, no signal was seen for negative – water control (data not shown).

In order to verify the presence and amplifiability of the plant DNA in the extracts, primers: plant1 and plant2 specific to plant chloroplast genome conserved sequences were used based on the previous studies [Datukishvili et al., 2007; Kutateladze et al., 2007]. Gel electrophoresis of PCR products revealed expected one amplicon in size approximately 400-500 bp for all plant DNA templates as was expected, no amplification signal was seen for negative water controls (data not shown). The results indicate high amplification quality of the DNA extracts.

In this study, detection of the 35S promoter and NOS terminator was applied for screening genetically modified organisms, because they are present in most of the authorized genetically modified plants, correspondingly use of these regulatory elements as targets allows to detect a vast number of transgenic plants. Especially CaMV 35S is the most popular promoter regulating new

inserted gene expression in both Roundup Ready soya and Bt-176 maize. PCR performed by the primer pair 35S1/35S2 specific for CaMV 35S promoter gave the expected amplicon in size of 195 bp when GM soybean and GM maize (including 0.1% GMO) genomic DNA was used as template, no PCR fragment was seen for non-GM plant samples and water control (Fig. 1 A, B).

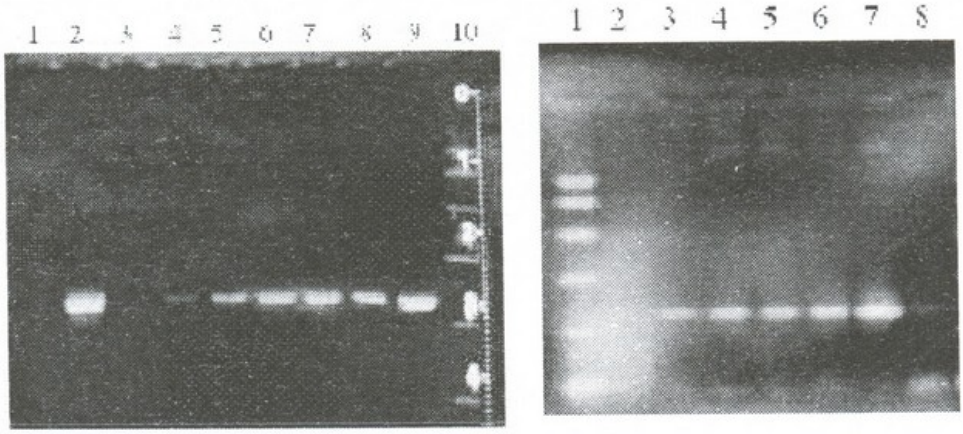

Fig. 1. GMO screening by PCR using CaMV 35S promoter-specific primer pair 35S1/35S2. PCR product in size of 195bp. **A**. Roundup Ready soy bean powder set: lane 1. negative water control; lanes 2 and 9 - 100% RRS; lane 3 - blank, lane 4 - 0.1%, lane 5 - 0.5%, lane 6 - 1%, lane 7 - 2%, lane 8 - 5%. **B**. Bt-176 maize powder set: lane 2 - blank, lane 3 - 0.1%, lane 4 - 0.5%, lane 5 - 1%, lane 6 - 2%, lane 7 - 5%, lane 8 - negative water control; lanes A10 and B1 - PCR markers: 1 kb, 750 bp, 500 bp, 300 bp, 150 bp, 50 bp (Promega).

The PCR primer pair NOS1 and NOS2 was used to detect NOS terminator that is present in Roundup Ready soya, but not in Bt-176 maize. These primers gave the expected amplicon in size of 180 bp when GM soybean (including 0.1% GMO) DNA was used as template, however no amplification signal was seen for non-GM soybean DNA and water control (Fig. 2). No PCR fragment was seen when maize DNA was applied as template, as expected (data not shown).

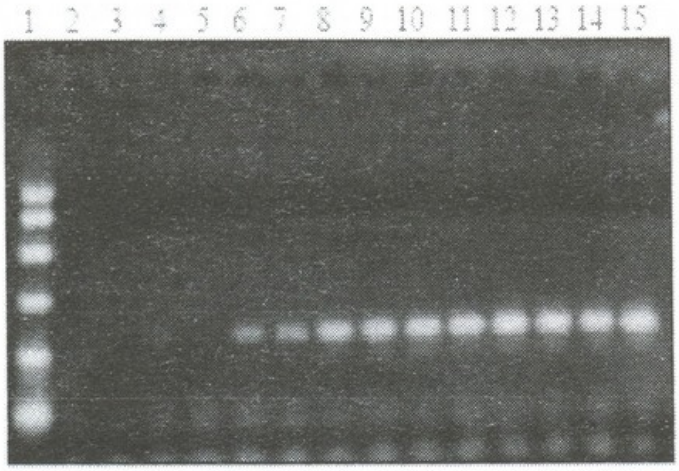

Fig.2. GMO screening by PCR using NOS terminator-specific primer pair NOS1/NOS2 of Roundup Ready soybean powder set; PCR product in size of 180 bp. lane 1 - PCR markers: 1 kb, 750 bp, 500 bp, 300 bp,150 bp, 50 bp (Promega); lanes 2, 3 - negative water control; lane 4, 5 - blank, lane 6, 7 - 0.1%, lanes 8, 9 - 0.5%, lanes 10, 11 - 1%, lanes 12, 13 - 2%, lanes 14, 15 - 5%.

The results obtained indicate that PCR tests, applied in this study, using primer pair 35S1/35S2 and NOS1/NOS2 are suitable for reliable and efficient detection of genetically modified organisms. These GMO-specific methods revealed detection sensitivity at least 0.1% of GM material. This value is better than 2% GMO detection limit, obtained previously for these primers

by Lipp et al. [Lipp et al., 1999]. In conclusion, qualitative detection methods, developed in this study, correspond to European regulatory requirements.

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გენეტიკურად მოდიფიცირებული ორგანიზმების თვისებრივი დეტექტირება

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რეზიუმე

შემუშავდა გენეტიკურად მოდიფიცირებული ორგანიზმების (გმო) სკრინინგის მეთოდები, რისთვისაც გამოყენებულ იქნა 0-5% Roundup Ready სოიას და Bt-176 სიმინდის სერტიფიცირებული საკონტროლო მასალების გამშრალი ფხვნილები, აგრეთვე 100% Roundup Ready სოიას თესლები. გენომური დნმ-ები ექსტრაგირებულ იქნა Qiagen DNeasy მცენარის მინი კრებულით. დნმ-ების ამპლიფიკაციის თვისება შემოწმდა მცენარის სპეციფიკური პოლიმერაზული ჯაჭვური რეაქციით (პჯრ), რომელიც შეესაბამება ქლოროპლასტის გენომის კონსერვატიულ თანმიმდევრობებს. 35S პრომოტორისა და NOS ტერმინატორის შესაფერისი პჯრ-ებით გამომჟღავნდა ტრანსგენური მასალის არსებობა გმო-ს შემცველ ყველა სერტიფიცირებულ საკონტროლო მასალაში. ამპლიფიკაციის სიგნალი არ გამოჩნდა უარყოფით ნიმუშებში, როგორიცაა წყალი და არამოდიფიცირებული სინჯი. სკრინინგის მეთოდებისთვის მიღწეულ იქნა ძლიერი მგრძნობელობა, როგორიცაა 0.1% გმო. მიღებული შედეგები აჩვენებს, რომ ამ კვლევაში აღწერილი, დნმ-ზე დაფუძნებული ანალიზებით შესაძლებელია გენეტიკურად მოდიფიცირებული ორგანიზმების ზუსტი, მგრძნობიარე და სწრაფი თვისებრივი დეტექტირება.

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B-FRUCTOFURANOSIDASE OF THE MUTANT STRAIN *PENICILLIUM CANESCENS* AME-85

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Abstract

β-Fructofuranosidase isolated from the culture liquid filtrate of the mutant strain *Penicillium canescens* AME-85 was purified up to electrophoretic homogeneity through precipitation by organic solvent, ion-exchange chromatography, and rechromatography on CM-cellulose column. Homogeneity of the enzyme was confirmed by electrophoresis on 8% PAAG with and without SDS. Specific activity of highly purified preparation was 540 U/mg protein, molecular weight - 148 kDa, temperature optimum - 50°C, pH optimum using 0,25% sucrose as a substrate – 5.0-5.3 and K_m - 11×10^{-3} M. The enzyme represents a glucoprotein and contains 6.0% carbohydrates. EDTA and PCMB did not inhibit the activity of the enzyme.

Key words: *β*-Fructofuranosidase, fungus, *Penicillium canescens.*

Introduction

β-Fructofuranosidase (*β*-D-fructofuranoside-fructohydrolase, invertaseE.C.3.2.1.26) catalyzes the cleavage of sucrose to glucose and fructose. Today, *β*-fructofuranosidase is widely used in food processing, especially in manufacturing jams and candies [Turkiewicz et al., 2005].

β-Fructofuranosidase is widely spread within plant and animal world [White et al., 1981; Belcarz et al., 2002]. Microorganisms of various taxonomic groups: yeast [Barnett, 1981; Ray et al., 1989], micromycetes [Kirillova et al., 1989; Fenice et al., 1997], bacteria [Hernalsteens et al., 2008], have ability to biosynthesize this enzyme.

The goal of the present work was isolation of highly purified *β*-fructofuranosidase preparation from *Penicillium canescens* AME-85 and study its properties.

Materials and Methods

A culture of the mutant strain of the fungus *Penicillium canescens* AME-85 was grown for 72 h at 28-30°C either in 750-ml flasks in a shaker or in automated 30-l fermenters (Marubishi, Japan) in the medium containing 30g/l soybean flour, 15 g/l $NaHPO_4 \cdot 12H_2O$, 2 g/l $(NH_4)_2SO_4$, 0.5 g/l KCl, and 0.15 g/l $MgSO_4 \cdot 7H_2O$. The commercial-grade preparation was precipitated from the filtrate of fungal culture liquid by an equal volume of ethanol cooled to -20°C. The precipitate was collected by centrifugation, dialyzed, lyophilized, and used as a commercial-grade preparation for practical application and further purification.

The purification proceeded as follows: the preparation (2 g) was dissolved in 20 ml of 0.05 M acetate buffer pH 4.5; the insoluble fraction was removed by centrifugation, and the supernatant was dialyzed over 24 h against the same buffer. The dialyzed protein solution (30 ml) was loaded on a CM-column (Whatman, Great Britain; 2.6x35 cm) equilibrated with 0,05M acetate buffer pH 4.5. Proteins were eluted from the column by a linear NaCl concentration gradient: elution rate was 24 ml/h; fraction volume - 8 ml. Active fractions were pooled, dialyzed against 0.05M acetate buffer pH 4.5, and recromatographed on a CM-column (1.6x35cm) equilibrated with 0.05M acetate buffer in a pH gradient of 4.3 to 6.2. Active fractions were pooled together, dialyzed against 0.05 M acetate buffer (pH 4.6) and employed for the study of physical-chemical properties of the enzyme.

Activity of *β*-fructofuranosidase was determined according to the increase in the content of reducing substances during hydrolysis of 0.25% sucrose solution at 30°C and pH 5.2 [Somogyi, 1952]; xylanase activity - using xylan as a substrate [Bailey et al.1992]. The activity of *β*-galactosidase was determined using *o*-nitrophenyl-*β*-*D*-galactopyranoside [Kuby et al.,1953].The reaction mixture (1 ml) containing 0,8 ml of phosphate-citrate buffer pH 4.2 supplemented with 125 mM of the substrate and 0.2 ml of the enzyme solution was incubated at 30°C for 15 min; 1 ml of 1 M Na_2CO_3 was added to stop the reaction. Produced *o*-nitrophenol was determined by optical absorption at 420 nm [Kuby et al., 1953]. Activity of *a* -galactosidase was determined using *p*-nitrophenyl-*a*-*D*-galactopyranoside (PNPG) [Zaprometova, 1986]. The reaction mixture containing 0,1 ml of 4mM PNPG (substrate), 0.2 ml of 0,05 M acetate buffer pH 4.5 and 0.1 ml of enzyme solution was incubated for 10 min at 37°C.The reaction was stopped by addition of 3 ml of 1 M Na_2CO_3, and the *p*-nitrophenol produced was measured at 405 nm.

Amount of enzyme required for hydrolysis of 1 mM of the substrate over 1min under standard conditions was taken as one unit of activity.

Protein content was determined either by the Lowry method [Loyry et al., 1951] or by optical absorption at 280 nm; total amount of carbohydrates in the protein was determined by the anthrone technique [Roe, 1951]. Optical absorption of the chromogen solution was recorded at 630 nm with a FEK-56 M instrument (Russia). The content of carbohydrates was calculated from the calibration curve obtained with glucose.

Disc-electrophoresis was carried out in 7.5% PAAG in Tris-glycine buffer (pH 8.3) [Davis, 1964]. Electrophoresis in plates of 8% PAAG with SDS in a two-phase system by Shapiro's modified method [Shapiro et al., 1967; Weber et al., 1969] was used to determine the molecular weight of the protein. The volume of the sample (containing 0.2 mg of the protein) loaded on the plates was 30 ml bovine serum albumin (BSA, 68 kDa), peroxidase (44 kDa) and lysozyme (14kDa) were used as markers.

Results and Discussion

At the first step of purification the filtrate of the culture liquid of the mutant strain *Penicillium canescens* AME-85 *β*-fructofuranosidase, *a*- and *β*-galactosidase and xylanase were used to obtain a commercial-grade preparation. Precipitation with organic solvents (alcohol and acetone), fractional precipitation with ammonium sulfate, and ethanol precipitation were employed to extract *β*-fructofuranosidase from the filtrate (500 ml of the filtrate was used). The results of these experiments are listed in Table 1. Precipitation with equal volume of ethanol proved to be optimal method because it concentrated the filtrate but preserved the activities of virtually all enzymes. The commercial-grade preparation obtained through precipitation with equal volume of ethanol displayed high activity, typically 12500-13000U/g preparation, and stability during storage at 4°C for one year.

A dialyzed solition of commercial-grade preparation was loaded on a CM column. Activities of the enzymes and protein content were determined in the resulting fractions. All four enzymes contained in the initial preparations were separated at this stage: xylase (fraction I), β-fructofuranosidase (fraction II), α-galactosidase (fraction III), and β-galactosidase (fraction IV).

Data on the chromatography on the CM column with NaCl concentration gradient are presented in Table 2. The specific activity of β-fructofuranosidase was 52.2 U/mg protein; purification – 22.5 fold. From 55-75% of xylanase, *a*- and β-galactosidase were collected with a 3- to 4-fold purification. The fraction II containing 75% of the entire β-fructofuranosidase loaded on the column, was dialyzed against 0,05 M acetate buffer pH 5.2 over 24 h and loaded on the CM column equilibrated with the same buffer. Proteins were eluted from the column by a pH gradient. One protein peak displaying only a β-fructofuranosidase activity was obtained. Specific activity of the fraction amounted to 540 U/mg protein (using 0.25% sucrose solution as a substrate). The data on the enzyme purification are listed in Table 3. The enzyme purified 45-fold; the activity yield was 36% of the total β-fructofuranosidase present in the culture liquid filtrate.

PAAG disc-electrophoresis of the purified β-fructofuranosidase yielded one protein band. Electrophoresis with SDS resulted in one band with a molecular weight of 148 kD. Occurrence of the only band in the SDS-electrophoresis suggests that the enzyme does not dissociate into monomers. The carbohydrate content in the homogeneous preparation from *P.canescens* AME-85 was determined by the anthrone method; it amounted to 6.0%.

Several properties of the β-fructofuranosidase (with a specific activity of 540.0 U/mg protein) were studied. The pH optimum of the enzyme was 4.0-4.2 using 0.25% sucrose solution as a substrate. The enzyme was stable over 48 h at room temperature in the pH range of 3.5-6.5; it retained 40% activity at pH 3.0 and completely lost the activity at pH 7.0.

The effect of temperature on the activity and stability of the enzyme was studied. The enzyme exhibited maximum activity when incubated over 15 min at 50°C using 0.25% sucrose solution as a substrate. The enzyme retained its activity over 4 h at 50°C, 20% of initial activity was retained after 10 min at 65°C and the enzyme was completely inactivated over the same time at 70°C. At room temperature, the enzyme retained its activity without loss over seven days. These data indicate a considerable thermal stability of β-fructofuranosidase from *P.canescens* AME-85. Under optimal conditions, the Km value determined from Lineweaver-Burk plots was 11×10^{-3} M using 0.25% sucrose solution as a substrate.

Highly purified β-fructofuranosidase (0.1mg protein/ml) and a commercial-grade preparation (0.1mg protein/ml) were used to study the effect of metals and several organic compounds on β-fructofuranosidase activity. The samples were previously dialyzed against 10^{-2} M EDTA and distilled water.

The effects of cations (Cu^{++}, Mn^{++}, Co^{++}, Ni^{++}, Ba^{++}, Na^{+}, K^{+}, Ca^{++} and Hg^{++}) at a final concentration of 10^{-3} M EDTA and PCMB ($0.2x10^{-3}$ over 24h) were studied. The enzyme retained its activity after dialysis. The isolated and purified β-fructofuranosidase from *P.canescens* AME-85 had much in common with the enzyme of micromycetes. The standard activity of the commercial-grade preparation obtained by precipitation with an equal volume of alcohol was 12500-13000 U/g, 3 times higher than the activity of the similar preparations isolated from other micromycete cultures [Nguyen et al., 1999]. The preparation was stable during storage and could be purified to homogeneity by simple purification methods yielding high activity levels (36%). Similar to most fungal β-fructofuranosidases, β-fructofuranosidase of *P.canescens* AME-85 had the activity optimum in the acid pH range, was stable at pH 3.5-6.5 and was thermally stable at 55°C. Neither EDTA nor PCMB affected the enzyme activity.

Thus, β-fructofuranosidase of *P.canescens* AME-85 displays considerable thermal and acid stability. Highly purified forms of the enzyme are likely to find their application in various branches of industry, and analytical biochemistry.

Table 1. Characterization of commercial-grade preparation obtained by various methods

Fraction	Protein		Enzymatic activity							
			β-fructofuranosidase		β-galactosidase		α-galactosidase		xylanase	
	mg	%	E	%	E	%	E	%	E	%
Filtrate	5000	100	14500	100	30000	100	2000	100	600	100
Fractionation, 60-100% saturation with (NH4)2SO4	600	12	12325	85	13500	45	1140	57	132	22
Precipitation by 1,5 volume of acetone followed by 4 volumes of ethanol	1500	27	10440	72	24900	83	1700	85	354	59
Precipitation by 1 volume of ethanol	1100	22	13630	94	26700	89	1820	91	528	88

Table 2. Characterization of the protein fractions collected from CM-cellulose column (NaCl gradient)

Fraction	V1	Protein		Enzymatic activity							
				β-fructofuranosidase		β-galactosidase		α-galactosidase		xylanase	
		mg	%	E	%	E	%	E	%	E	%
Commercial-grade preparation*	40	210	100	14900	100	45800	100	3850	100	1575	100
I (1 - 15) **	120	3	1	149	1	159	<1	31,5	1	787,5	50
II (16 - 30)	120	9	4	6705	45	457	1	1,5	<1	39,5	2
III (31 - 45)	120	56	27	2980	20	1374	3	2541	66	0,5	<1
IV (46 - 60)	120	76	36	447	3	36640	80	53,9	1	0	0
Total		144	59	10281	69	38630	84	2627,9	68	827	52

* The commercial-grade preparation (2 g) was extracted from 500 ml of the culture liquid filtrate.
** 1 - 60 are the numbers of the tubes containing the 8-ml fractions collected from the column.

Table 3. Purification of the β-fructofuranosidase of Penicillium canescens mutant strain

Stage	Activity, U	Yield %	Protein mg	Specific activity U/mg protein	Purity
Culture liquid filtrate	15000	100	1250	12	1
Precipitation by 1 volume of ethanol	14100	94	313.3	45	3.75
CM-cellulose (NaCl gradient)	9750	65	52.2	270	22.5
CM-cellulose (pH gradient)	5400	36	8.7	540	45

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Penicillium canescens AME-85 მუტანტური შტამის β-ფრუქტოფურანოზიდაზა

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დურმიშიძის ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტი

(მიღებულია 20.01.2009)

რეზიუმე

Penicillium canescens AME-85 მუტანტური შტამიდან გამოყოფილი β-ფრუქტოფურანოზიდაზა გაწმენდილ იქნა ელექტროფორეზულად ჰომოგენურ მდგომარეობამდე კულტურალური სითხის ფილტრატიდან ორგანული გამსხნელის დალექვით და ორმაგი იოცვლადი ქრომოტოგრაფიით CM- ცელულოზაზე. ფერმენტის ჰომოგენურობა დადასტურებულ იქნა ელექტროფორეზით 8%-იან პოლიაკრილამიდ გელში SDS-Na-ით და მის გარეშე. ხვედრითმა აქტივობამ მაღალგაწმენდილი პრეპარატისა შეადგინა 540 ერთ/მგ ცილაზე. მოლეკულური წონა 148000 დალტონი, ტემპერატურული ოპტიმუმი 50°C, pH ოპტიმუმი 5.0-5.3 სუბსტრატად 0.25% საქაროზის ხსნარის გამოყენების შემთხვევაში, Km - 11×10^{-3}: ფერმენტი წარმოადგენს გლიკოპროტეიდს და შეიცავს 6% ნახშირწყლებს. EDTA და PCMB არ აინჰიბირებენ ფერმენტის აქტივობას.

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POLLINATION AND MALE CONE MORPHOMETRY IN *CEDRUS* SPECIES FROM THE COLLECTION OF TBILISI BOTANICAL GARDEN.

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Abstract

Microsporangia dehiscence, timing and duration of pollination have been studied in 3 *Cedrus* species growing at the Tbilisi Botanical Garden. Observations conducted in 2008 revealed that pollination time overlapped in *C. atlantica*, *C. libani* and pollen release starts about one month earlier than in *C. deodara*. To characterise and discriminate species, which are genetically closely related, morphometric analysis of microstrobili was carried out, using 7 morphological parameters. Morphological traits of male cones differ enough to be useful for identification of Himalayan cedars from species of Mediterranean origin. The program SPSS 13.0 was employed for statistical evaluation of measurement results by means of Principal Component Analysis (PCA) and Discriminant Function Analysis (DFA).

Key words: *Cedrus libani, C. atlantica, C. deodara,* pollination, male cone development, morphometry.

Introduction

The true cedar genus belonging to Pinaceae family includes four species: *Cedrus libani* A. Rich. - Cedar of Lebanon, *C. atlantica* Man. - Atlas cedar *C. brevifolia* Hook. - Cyprian cedar and *C. deodara* (Roxb.) G. Don. - Himalayan cedar [Farjon, 2001]. Present geographic distribution of the genus is limited to Morocco and Algeria for *Cedrus atlantica*; to Lebanon, Syria and Turkey for *C. libani*; to Cyprus for *C. brevifolia* and to Himalayan Mountains for *C. deodara* [Debazac, 1964; Vidacovic, 1991].

Besides providing economical benefits from timber production in countries of origin, *Cedrus* species are of particular importance for landscaping in most temperate regions of the world due to outstanding ornamental features of mature trees [Chaney, 1993].

There are 3 species [Manjavidze, 1950, Skhiereli, 1952] in the collection of the Tbilisi Botanical Garden: *C. atlantica, C. libani,* and *C. deodara* (Fig. 1, a, b, c). *C. deodara* is very popular ornamental tree widely planted in Georgia for its beauty of form and grandeur in old age and adaptability to a wide range of climatic and soil conditions. In contrast to this, trees of *C. atlantica* are rare and *C. libani* is practically absent as garden ornamental, despite a statuesque habitués of adult specimens, and thus deserving merits for their use in landscaping. Successful collection and utilization of seeds to produce the effective means for artificial propagation is an appropriate tool that should be used in order to raise seedlings for enrichment *Cedrus* plantings. Formation of the fertile seed being able to germinate is essential for predictable seedling

establishment. The reproductive cycle is an important part of cedar's life cycle that involves a chain of developmental phases from the initiation of generative structures to seed germination. Pollen production is a key attribute for the sexual reproductive success.

The aim of this study was to investigate the development and maturation of male reproductive organs of *C. libani, C. atlantica* and *C. deodara* growing at the Tbilisi Botanical Garden. As these tree species are closely related genetically [Panetsos et al., 1992; Dagger-Harrat et al., 2001], another aim of the study was to reveal species-specific morphologic features of microstrobili and timing and duration of pollination.

Material and Methods

Male cones were collected at principal stages of the development from May to October 2008. Observations were made under a stereomicroscope DV 4 (Karl Zeiss, Germany). Dehiscent and near dehiscent microstrobili were cut into sections using a razor blade. Microsporangia were macerated with a syringe needle, released pollen grains were cleared in Herr's solution [Herr, 1971] and examined under light microscope Polivar (Reichart, Austria) equipped with Nikon CoolPix 5000 digital fotocamera. Trees heights were determined with Abney Level (Breithaupt Kassel, Germany). Morphometric measurements of pollen grains were done using an ocular and stage micrometers. Microstrobili were measured in millimeters. Statistical analysis for evaluation of measurement results was undertaken with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Based on the Garden's records [Skhiereli, 1952], studies were conducted on the adult *Cedrus* trees over 100 year old. *C. libani* growing on the northern slope (41° 41′ 20′′N, 44° 48′ 19′′E) is strongly monoecious sample. The diameter of tree measured at breast height is 70 cm with a height up to 16 m and a broadly tabular shape. Female cones and microstrobili are widely scattered throughout the crown. They are located on separate branches, however, no preferential allocation of female and male cones in distal and proximal regions of the tree crown was observed. It seems that spatial isolation of male and female cones in monoecious conifers doesn't occur at least in case of *C. libani*. The general scheme of microstrobilus development is similar to that of other cedars that have been studied [Smith, 1923; Maheshvari, Bisvas, 1970; Farjon, 1990]. Pollen cone development extends over approximately 3 month. Microstrobilus is initiated around the brachyblast's apex at the end of May. At mid-June the first microsporophyll primordium becomes visible halfway up the cone axis. Subsequently microsporophylls are arranged in a tight helix around the pollen cone axis. Differentiation of microsporophylls occurs during approximately one month. Each microsporophyll develops two abaxial microsporangia. On the cross section of the premature microstrobilus (Fig. 1, d) microsporangia are well developed (Fig. 1, e). The middle part of the microstrobilus contains bigger microsporophylls, compared to basal and apical ones (Fig. 1, f). However, the stage of development of the pollen grain and its morphometric parameters is almost similar in the whole male cone. The data revealed that a strobilus took 2-3 days to dehisce completely probably due to variation in prevalent air temperature and relative humidity. As a rule bissacate pollen grains of *C. libani* are three-celled inside the near-dehiscent microsporangium. Before shedding the corpus is oblate, suboblate or oblate spheriodal (Fig. 1, g). During pollen development the microsporangial wall forms a slit of dehiscence on the lateral surfaces. As the pollen cones dry in autumn, the microsporangia split open along this slit releasing the pollen. Microstrobili were generally abundant and a quiet large pollen clouds were observed even in the light wind case. Pollination in *C. libani* started as much as 4 weeks ahead of the close neighbouring

C. deodara trees. We observed first pollen dehiscence on October 5. Pollination was completed on November 7. To compare, dehiscence in *C. atlantica* trees began about one week earlier (end of September, Fig. 1, i). It seems that self-pollination regularly occurred in the upper and middle branches of the *C. libani* tree crown. Phenologically synchronized development of the male and female reproductive structures was detected. The bisaccate, buoyant pollen grains adhere firmly to female cone scale surface. We observed several stages of pollen penetration into the megastrobilus. At first pollen lands on the abaxial surface of the ovuliferous scale (Fig. 1, j). Then pollen passes on the ovuliferous scales and rolls down towards the adaxial surface of the underlying ovuliferous scale. Finally pollen grain adheres into the ovule apex which is extended into the area between the ovuliferous scales and cone axis. (Fig. 1, k). Although the number of pollinated ovules was high especially in the middle parts of the megastrobili, we can suppose that the low percentage of germinated seeds (preliminary data) is due to self-pollination.

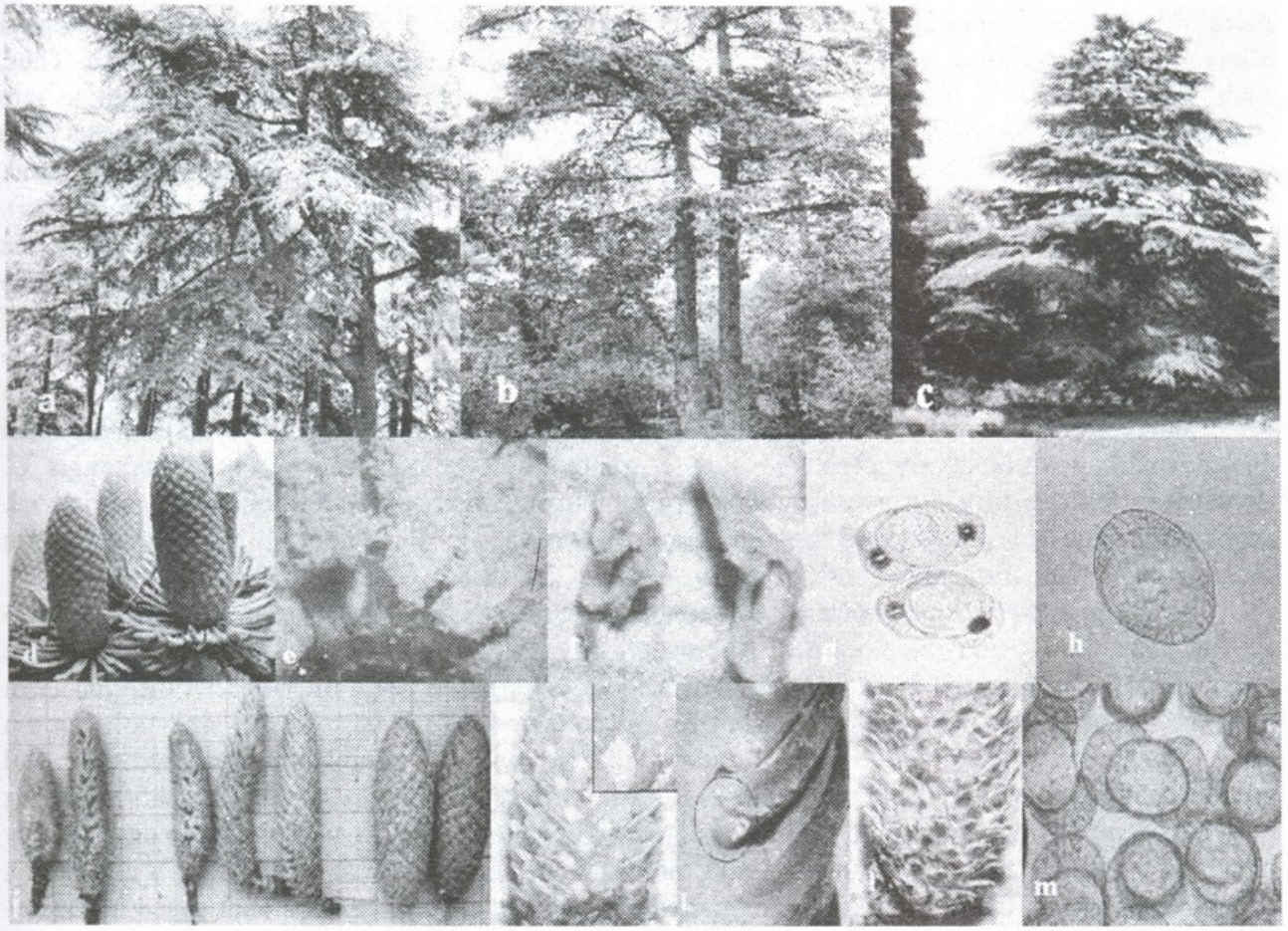

Fig. 1. a- *C. libani*, b - *C. atlantica*, c - *C. deodara* at the Tbilisi Botanical Garden, d - premature microstrobili of *C. atlantica*, 4/5 of natural size and pointed apex in the upper right corner, x 3.3, e - cross section of microstrobilus of *C. libani* before pollination, x 7.5, f - microsporophylles from the basal (left) and central (right) part of the dehiscent microstrobilus of *C. libani*, x 11, g - pollen grains of *C. libani*, x 265 and h - *C. atlantica*, x 360, i – from left to right: microstrobili of *C. libani*, *C. atlantica* and *C. deodara* in mid-October, 3/4 of natural size, j - macrostrobilus with pollen grains adhesed on the abaxial surface of the ovuliferous scales, x 4, the same in the upper right corner, x 12, k - pollen grains inside the macrostrobilus, x 23, l - microstrobilus of *C. deodara* at the peak of the pollination, x 3, m - pollen grains of *C. deodara* before microsporangia dehiscence, x 215.

Most of the *C. atlantica* trees at the Tbilisi Botanical Garden are monoecious. Numerous male strobili occurred in the lower and middle branches of the crowns, while less abundant macrostrobili develop at the top of the trees. There are little morphological differences between *C. atlantica* and *C. libani* [Maheshwari, Biswas, 1970; Farjon, 1990]. It must be mentioned that *C. atlantica* is so closely related to *C. libani* that some habitual characteristics help differentiate the species. In general, *C. atlantica* is distinguished from the *C. libani* by a taller crown, less densely arranged branchlets and silvery blue needles (f. *glauca*). Studied sample of *C. atlantica* is about 19 m tall, with massive stem bifurcated at 70 cm from the tree base. Pollination mechanism agrees very closely with that of *C. libani.* However the time and duration of the pollination is slightly distinguished. Thus pollination in *C. atlantica* started at 30 of September (air t 21° C), whereas in *C. libani* first visible microsporangia dehiscence occurred in 5 of October (air t 19° C).

C. deodara trees planted in Himalaya sector in 1936 have strongly performed sexualization type i.e. the differences in the sex ratio of the individual trees in *C. deodara* are rather more obvious than it could be supposed for monoecious tree species. The evidence for preferential allocation of cones to particular tree types is actually presented in these monoecious trees. Thus, male trees develop microstrobili almost at all branches except for tree apex, where single rare megastrobili occur. In contrast to that female trees develop numerous megastrobili, but no one male cone has been observed. Studied Himalayan cedars belong to mature trees and are over 80 year old. Pollination begins with the opening of microsporiphylls toward the outside of cone axis (Fig. 1, l). The dehiscence of microsporophylls proceeds, as a rule, from the broader central part to the narrower basal end of the microstrobilus. Microsporangia form a longitudinal slits and massive pollen emission occurs (Fig. 1, m). Our observations have also revealed that in the majority of strobili microsporangia do not dehisce simultaneously even in case of two lobes of the same microsporangium. Microstrobilus took, on average, 2-3 days to dehisce completely, depending on the temperature and atmospheric humidity conditions. It seems that high temperatures hastened pollen shedding while low temperatures and wet weather delayed dehiscence of microsporangia. *C. deodara* is less sensitive to average temperatures during microsporangia dehiscence period i.e. pollination time doesn't depend on temperature decrease in December. Similar results were obtained from the natural stands of *C. deodara* in India [Khanduri, Sharma, 2002].

Because these three species are genetically closely related [Panetsos et al., 1992, Dagger-Harrat et al., 2007] and their pollination mechanisms are very similar, we tried to reveal some minor differences between them. Morphometric analysis revealed slight interspecific differences in some of the morphological characteristics of male cones (pollen grain corpus size, pollen grain total size, microstrobilus length, microstrobilus width, microsporophyll length and microsporangium length). The morphological variation of 62 samples was then analyzed using principal component analysis and multivariate discriminant analysis. The factors were extracted by means of principal component analysis and the parameter settings were as follows: a correlation matrix was used and two factors were extracted to visualize the two dimensional space of macro- and micromorphological characteristics of microstrobili. In order to simplify the interpretation of the extracted factors, factor rotation was performed, during which orthogonal rotation method, Varimax, was used. The factor analysis indicates that traits such as: microstrobilus length, microstrobilus width, microsporophyll length microsporangium length and pollen grain total size have a significant factor loading with the first factor (48% of variance). Pollen grain corpus size is correlated with the second factor (22% of variance), meaning that this characteristic is of a minor importance for the species identity. The study of the development and maturation of microstrobili revealed that *C. libani C. atlantica* and *C. deodara* are significantly differing in morphometric characteristics of male cone, namely pollen size (Tab.1). The mean size of mature pollen grains of *C. libani* and *C. atlantica* measured in equatorial plane of vital pollens cleared in Herr's solution is 76 ± 8 μm. The pollen size of *C. deodara* is bigger (86 ± 3 μm) and differs from these two species

significantly ($p<0.001$; 95% confidence interval). A discriminant analysis was then performed on the morphometric data of microstrobili to determine the usefulness of selected morphological parameters for the description and separation of studied species (Fig. 2). The separation of *C. deodara* from the *C. libani* and *C. atlantica* is obvious. The overlap between *C. libani* and *C. atlantica* is also evident. In analyzing the data one can see that classification of cases using discriminant analysis yielded a correct classification of about 90% of original grouped cases (Tab. 2). Data obtained indicate the fact that based on overall 7 characters the studied species could differ in their micro-morphological features. Our results are in accordance with the data on phylogeny and biogeography of *Cedrus*: the separation of *C. deodara* and the close relationships among the Mediterranean cedars are corroborated by the sequence analysis of cp- and mt-DNA fragments [Qiao et al., 2007]. This indicates that selected morphological characters can be considered as important variables that differentiate microstrobili of studied *Cedrus* species.

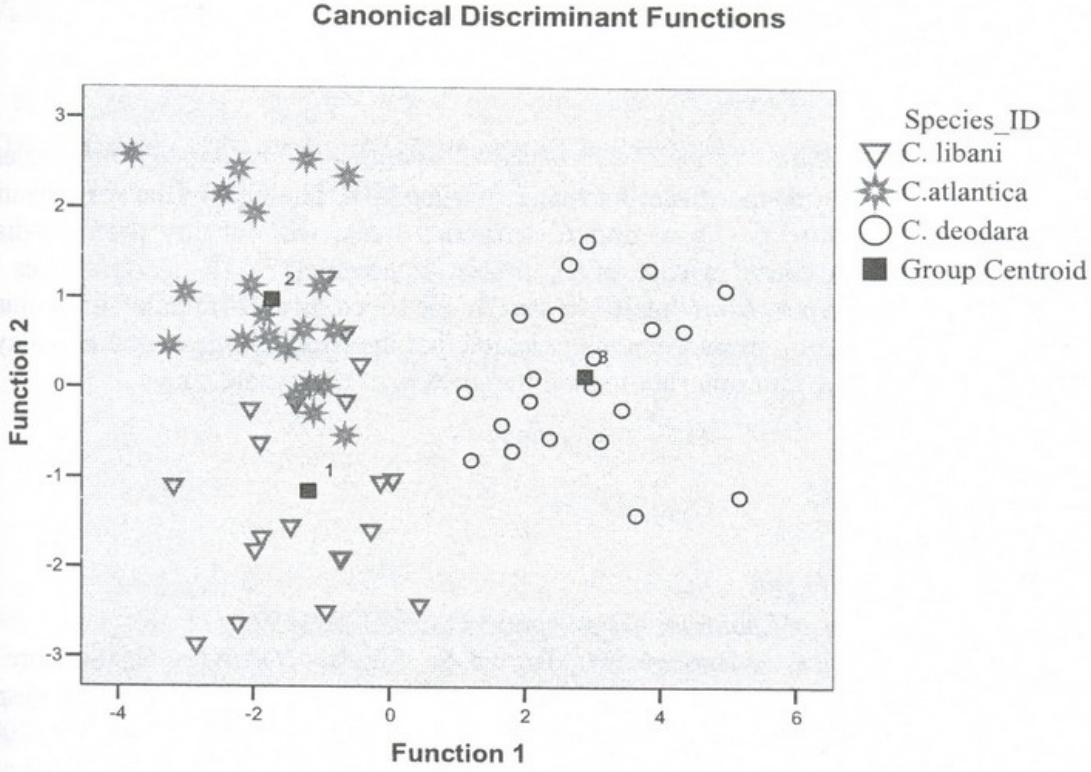

Fig. 2. Scattered diagram of the studied species on the two discriminant functions

Table 1. Pollen grain total size analyzed by one-sample t-test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
C.libani	39.109	19	.000	.075350	.07132	.07938
C.atlantica	41.392	21	.000	.076818	.07296	.08068
C.deodara	121.886	21	.000	.086000	.08453	.08747

Table. 2. Classification of cases using discriminant analysis

Classification Results[a]

			Predicted Group Membership			
		Species_ID	1	2	3	Total
Original	Count	C. libani	16	4	0	20
		C. atlantica	2	19	0	21
		C. deodara	0	0	21	21
	%	C. libani	80.0	20.0	.0	100.0
		C. atlantica	9.5	90.5	.0	100.0
		C. deodara	.0	.0	100.0	100.0

a. 90.3% of original grouped cases correctly classified.

Conclusions

Pollination time and duration of the *C. libani* and *C. atlantica* is overlapped. Pollen release in these two species starts about one month earlier than in *C. deodara*. The type of the sexualization is strongly monoecious in mature *C. libani* and *C. atlantica* trees, without any proximo-distal separation of male and female cones in case of *C. libani*. In contrast, in *C. deodara* trees we observed a diversity of sexual types, from "male" to strictly monoecious and "female" individuals. Statistical analysis of morphometric measurements revealed that the Mediterranean and Himalayan species significantly differ by the micromorphological parameters of their male cones.

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დამტვერვის შესწავლა და მიკროსტრობილების სტრუქტურული ელემენტების მორფომეტრული ანალიზი თბილისის ბოტანიკური ბაღის კოლექციის გვარ *Cedrus*-ის სახეობებში.

შაქარიშვილი ნ., ხმალაძე ს., ტუღუში კ.

თბილისის ბოტანიკური ბაღი და ბოტანიკის ინსტიტუტი

(მიღებულია 06.01.2009)

რეზიუმე

შესწავლილია მამრობითი გენერაციული სფეროს განვითარება თბილისის ბოტანიკური ბაღის გვარ *Cedrus*-ის კოლექციის სახეობებში: *C. libani, C. atlantica* და *C. deodara.* დამტვერვის ვადები და ხანგრძლივობა დადგენილია 2008 წლის დაკვირვებების საფუძველზე. ჩატარებულია მიკროსტრობილის სტრუქტურული ელემენტების მორფომეტრული ანალიზი 7 მორფოლოგიური ნიშნის მიხედვით. გენეტიკურად ახლომონათესავე სამივე სახეობის დახასიათებისა და დაჯგუფებისთვის მონაცემები დამუშავებულია მთავარი კომპონენტებისა (PCA) და დისკრიმინანტული ფუნქციების ანალიზის (DFA) საშუალებით. გამოთვლები შესრულებულია სტატისტიკური პროგრამის **SPSS 13.0** მეშვეობით. შერჩეული მორფოლოგიური მახასიათებლების გამოყენებით შესაძლებელია ხმელთაშუაზღვეთის სამხრეთსა და აღმოსავლეთში გავრცელებული სახეობების (*Cedrus libani*-სა და *C. atlantica-s*) დაჯგუფება და ჰიმალაის დასავლეთ ნაწილში გავრცელებული *C. deodara*-ს გამოყოფა.

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GENETIC CHANGES INDUCED BY ACTION OF PESTICIDE ZINEB IN SEEDLINGS DEVELOPED FROM THE SOYBEAN SEEDS OF DIFFERENT AGE

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Abstract

The work aims at identifying genetic activity of pesticide zineb, on delayed soybean seeds of different age. One- two-, and three-year seeds of the soybean (*Glycine max*) genetic line L65-1237 have been used in the trial. The seeds were subjected to the action of agent zineb of various concentrations (0.02%, 0.04%, 0.06%, 0.08% and 0.1%) at 24-hour exposure. Induced somatic mutations were registered on the heterozygote ($Y_{ii}y_{ii}$) seedling leaves. In the seedlings developed from one-year seed, zineb induced only mutation changes. The direct mutation induction was carried out with much higher frequency in comparison with the back mutation. In the seedlings developed from two- and three-year seeds, zineb induced both direct and back mutations with a higher frequency as compared with one-year seed seedlings. The mitotic crossing-over occurs with high frequency. The "dose-effect" phenomenon was expressed. The age growth was found to be positively correlated with the mitotic crossing-over induction which is a result of the genetic material destabilization.

Key words: soybeans, pesticide, plant aging, zineb

Introduction

Aging is one of the fundamental properties of onthogenesis. In the course of biological aging, the vital processes taking place in the organism gradually deteriorate and weeken. The organism reveals less adaptivity to the habitat. The operation of the repairing system becomes less effective with age. The cumulation of spontaneous mutations takes place in the organism. All this induces destabilization of the genetic material and affects the normal expression of genes [Potapenko, Akifyev, 2003; Zhmylev, 2006].

In contrast to animals, the programmed mechanism of aging is not characteristic of plants. Many aging-initiation factors have been identified in plants [Hayflick, 2007]. The environmental mutagenic factors may also be treated as plant-aging provoking factors. They contribute to the cumulation of mutations and enhance destabilization of the genetic material. In this regard the genetically active xenobiotics, particularly pesticides are noteworthy. The living system has not encountered with such compounds in its evolutionary process and has not obtained its destructive enzymatic system. The genetically active pesticides affect the genetic system of a living organism and induce its modification.

Materials and Methods

The genetic line L65-1237 derived in soybean (*Glycine max*) by American scientists [Vig, Paddock, 1970], the origin of which is described in our earlier publications [Baratashvili et al., 2003; 2008; Kadagishvili et al., 2006], has been used in our trials. The trials were conducted on seeds developed from a heterozygote plant ($Y_{ii}y_{ii}$). The Y_{ii} allele is semidominant; it controls the chlorophyll synthesis. Three phenotype plant classes develop from the seed: green ($Y_{ii}y_{ii}$), light-green ($Y_{ii}y_{ii}$), and yellow ($Y_{ii}y_{ii}$), in the 1:2:1 proportion.

The pesticide zineb $(C_4H_6N_2Zn)n$ is a polymer. It is widely used in Georgia against fungal diseases. One-, two-, and three-year soybean seeds were subjected to the agent diluted in the distilled water in the concentrations of 0.02%, 0.04%, 0.06%, 0.08%, and 0.1%. 100 air-dry seeds used to be treated with the agent of specific concentration during 24 hours. Following the procedure, the seeds were washed in the running water during 4 hours and sawn in wooden boxes, in the established procedure [Vig, Paddock, 1970].

The heterozygote (light-green) plant seedlings were analyzed, because any somatic genetic modifications could be registered thereon. The plant was analyzed at one-simple and two-complex leaf stages. Spots appearing on the upper leaf surface were microscopically analyzed (magnification 2x10); the obtained results were statistically processed [Plokhinski, 1970].

Results and Discussion

A study of spontaneous and induced mutation processes in seeds of different ages makes it possible to speak on the genetic structure stability. It represents one of the most suitable models in revealing the plant-aging provoking factors [Zhmylev, 2006; Hayflick, 2007]. It is known that chromosomal mutations are induced with high frequencies on delayed seeds by super mutagens [Dubinin, 2000]. The genetic changes on delayed seeds induced by the effect of xenobiotics, in particular pesticides, are less studied.

The results obtained in the experiments have demonstrated genetic activity of the pesticide zineb (see Table 1). On both the simple and complex leaves of the light-green ($Y_{ii}y_{ii}$) seedlings grown from the one-year seed somatic mutations were induced. Both the direct (yellow spot) and back (green spot) mutations developed. The high dose of the studied agent led to the induction of mitotic crossing-over (double spot, half-green and half-yellow) at a low frequency. Zineb used to induce at a higher frequency yellow spots (direct mutation) than green ones (back mutation). At the same time, the "dose-effect" phenomenon was well identified. A rather low background of spontaneous mutation induction needs to be mentioned, constituting 0.28 spot per leaf on the average.

The control trial versions developed from the delayed seeds demonstrated the growth of the spontaneous mutation background. In the seedlings grown from the two-year seeds this indicator made 0.33, and 0.69 in the seedlings grown from the three-year seeds (see Tables 2 and 3).

In the plants developed from the delayed seeds zineb induced significant genetic changes, which should be associated with the genetic system destabilization. The agent caused a growth in the number of both the yellow and green spots. Upon induction of yellow spots the "dose-effect" phenomenon was well expressed. The frequency of back mutations induced in the plants developed from the two-year seeds was found to be approximately the same as in the one-year ones. All the doses of the agent induced double spots in the plants grown from the two-year seeds with the same low frequency. As has been mentioned, it was developed as a result of the mitotic crossing-over. The mitotic crossing over was induced at a high frequency (0.11-0.46) in the seedlings grown from the three-year seeds and the "dose-effect" phenomenon was expressed.

Table 1: Zineb-induced genetic changes in plants grown from one-year seeds

Substance concentration, %	Number of analyzed leaves	Total number of spots	Average number of spots per leaf			
			yellow	green	double	Total
0.02	101	69	0.48±0.01	0.20±0.02	0	0.68±0.03
0.04	103	72	0.49±0.02	0.21±0.03	0	1.70±0.04
0.06	103	72	0.49±0.02	0.21±0.03	0	1.12±0.03
0.08	116	148	0.96±0.01	0.32±0.03	0	1.28±0.02
0.1	99	162	1.12±0.01	0.49±0.03	0.03±0.03	1.64±0.03
Control	179	50	0.19±0.03	0.09±0.03	0	0.28±0.05

The genetic structure of germinal cells of the three-year seed has been found to be more sensitive to zineb, where the direct mutations used to be induced at a higher rate; in particular, the action of the 0.1% agent induced the appearance of 1.46 spots per leaf on the average (see Table 3).

The literary data indicate the increase of the frequency of chromosomal aberrations by the action of ionising radiation and chemical mutagens on the delayed seeds in comparison to the control. The mitotic disorders in the root meristem tissue developed from the seed were investigated [Valeva, 1969; Dubinin, 2000].

The effect of chemical mutagens and irradiation on the genetic system has been studied in the soybean plant seedlings developed from one-year seeds of the same genetic line. The irradiation is found to induce somatic mutations at a much higher rate than the chemical mutagens used in the trials. The induction of direct mutations took place at a higher rate as compared with the back mutations [Vig, 1974; Evans, Paddock, 1980]. The data obtained by these authors coincide with our data. The effect of neutrons and γ-irradiation on one-year seeds has been studied. The neutrons revealed much higher mutagenic and recombinogenic effect [Davronov, Zakharov, 1985]. If our data are compared to the radiation effect, it will be evident that radiation, as compared to zineb, is more effective in inducing both direct and back mutation, as well as mitotic crossing-over.

Table 2: Zineb-induced genetic changes in plants grown from two-year seeds

Substance concentration, %	Number of analyzed leaves	Total number of spots	Average number of spots per leaf			
			yellow	green	double	Total
0.02	122	119	0.64±0.04	0.31±0.02	0.04±0.03	0.98±0.02
0.04	101	99	0.67±0.01	0.29±0.03	0.03±0.02	0.98±0.03
0.06	103	138	0.92±0.02	0.37±0.01	0.05±0.03	1.34±0.05
0.08	107	145	0.93±0.02	0.38±0.03	0.05±0.02	1.36±0.04
0.1	93	158	1.07±0.04	0.56±0.03	0.07±0.02	1.70±0.02
Control	150	49	0.20±0.01	0.11±0.02	0.02±0.03	0.33±0.02

Table 3: Zineb-induced genetic changes in plants grown from three-year seeds

Substance concentration, %	Number of analyzed leaves	Total number of spots	Average number of spots per leaf			
			yellow	green	double	Total
0.02	120	125	0.51±0.02	0.42±0.03	0.11±0.03	1.04±0.03
0.04	110	149	0.69±0.03	0.53±0.04	0.03±0.02	1.35±0.02
0.06	91	157	0.93±0.01	0.60±0.01	0.20±0.03	1.73±0.04
0.08	93	207	1.13±0.05	0.81±0.04	0.29±0.03	2.23±0.03
0.1	90	256	1.46±0.04	0.92±0.03	0.46±0.04	2.84±0.03
Control	106	80	0.35±0.05	0.28±0.05	0.06±0.03	0.69±0.03

The same test system developed in soybean was used to study the effect of pesticide Ridomil on the delayed seeds. Ridomil lead to a high-rate induction of direct mutation in seedlings grown from delayed seeds, with the expression of the "dose-effect" phenomenon [Baratashvili,et al., 2008].

The results of our experiments have demonstrated the genetic structure destabilization with ageing with the resultant growth of the spontaneous mutation background and higher rate of mutation induction in the seedlings grown from the delayed soybean seeds.

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პესტიციდ ცინების მოქმედებით გამოწვეული გენეტიკური ცვლილებები სოიას განსხვავებული ასაკის თესლიდან განვითარებულ აღმონაცენებში

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რეზიუმე

გამოვლენილია სხვადსხვა ასაკის დაყოვნებულ თესლებზე პესტიციდ ცინების გენეტიკური აქტივობა. ექსპერიმენტში გამოყენებულია სოიას (Clycine max) გენეტიკური ხაზის L-65-1237 განსხვავებული ასაკის (ერთ, ორ და სამწლიანი) თესლები. თესლები დაექვემდებარა ცინების სხვადასხვა კონცენტრაციის (0.02%; 0.04%; 0.06%; 0.08% და 0.1%) პრეპარატის ზემოქმედებას 24 სთ. ექსპოზიციით. ინდუცირებული სომატური მუტაციები აღირიცხებოდა ჰეტეროზიგოტ ($Y_{II}y_{II}$) აღმონაცენთა ფოთლებზე. ერთწლიანი თესლიდან განვითარებულ აღმონაცენებზე ცინებმა გამოიწვია მხოლოდ მუტაციური ცვლილებები. პირდაპირი მუტაციების ინდუქცია გაცილებით მაღალი სიხშირით მიმდინარეობდა ვიდრე რევერსიების. ორ და სამწლიანი თესლიდან განვითარებულ აღმონაცენებში, ერთწლიანთან შედარებით ცინებმა მაღალი სიხშირით მოახდინა პირდაპირი რევერსების ინდუქცია. მაღალი სიხშირით მიმდინარეობდა მიტოზური კროსინგოვერი. მოქმედებდა ფენომენი „დოზა-ეფექტი“. ასაკის მომატებასთან დადებით კორელაციური დამოკიდებულებებში აღმოჩნდა მიტოზური კროსინგოვერის ინდუქცია, რაც გენეტიკური მასალის დესტაბილიზაციის შედეგია.

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FEATURES OF SPONTANEOUS AND INDUCED MUTANTS OF *CITRUS NOBILIS* KOVANO VASSE

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Abstract

Both, at spontaneous and induced mutations of *Citrus nobilis* Kovano vasse phenological and generative alterations take place. It concerns growth rate, leaf form, cutting morphology, fruit form and coloration, seminiferous and spicular characteristics, contents of C vitamin and sugar, early ripeness and other features. Bioproducts accumulated have potential of definite mutagenic effect on cell genetic system, which in turn increases capabilities of origination of spontaneous mutations of mandarin.

Key words: spontaneous and induced mutants, *Citrus nobilis* Kovano vasse, chromosome aberrations

Introduction

By climatic-geographic features subtropical region of Georgia is azonal and formation of its nature is affected by local geographic, climatic and orographic factors and therefore it is characterized by rather sharp changes. So, genetic studies of plants, and particularly citruses is necessary, which should be directed towards raising of adaptive and immune resistant varieties [Dolidze, 2003]. The most adaptive to subtropical conditions of Georgia and perspective in economic viewpoint is mandarin, and especially its early ripening varieties.

Level of natural adaptability of citruses is likely achieved its maximum of biological abilities. Therefore their genepool by using of conventional selective methods is hardly submitted to changes desirable for us. To receive valuable genetic structures new genetic methods are needed. Such methods enable to obtain new spontaneous and induced mutant forms and to study interesting ones, which have practical and theoretical significance for enrichment of citrus genepool [Dolidze, 2004].

The goal of our study was determination of the level of changes of some physiological and genetic characteristics of early cultivars of *Citrus nobilis* Kovano vasse.

Materials and Methods

The object of our study was 25 spontaneous and 58 induced mutants, which are received under the effect of chemical mutagens and irradiation.

Contents of C vitamin and sugar were determined according to [Ermakov et al., 1972].

Study of biomutagenic effect of leaf extract of sterile forms of mandarin was conducted according to [Diasamidze, 1995].

Obtained data were worked out statistically [Voznesenski, 1969].

Results and Discussion

Observations on the spectrum of changes of vegetative period of spontaneous and induced mutants of *Citrus nobilis* Kovano vasse show that in spite of differences in ways of formation of mutants, in the process of their growth and development both, similar and distinct deviations are noted. In mutants detected according to vegetation period, alterations of growth periods, beginning and ending of florescence phenological phases etc., long term as well as short term vegetation periods are observed. Early and late flourishing, early and relatively late ripening forms were also recorded.

While spontaneous, as well as induced mutations changes of phenological and generative character occur. It concerns growth rate, leaf form, cutting morphology, fruit form and coloration, seminiferous and spicular characteristics, contents of C vitamin and sugar, early ripeness and other features. Studying of this problem assist in using those mutants for obtaining the earliest mandarin forms or forms distinguished by other characteristics.

Mandarin, because of its polymorphism, forms homologous variations very intensively, which differ from each other both, by single and complex features. At the same time, they are distinguished from the fruit of the initial variety by acidity, contents of C vitamin and sugar. Study of the results of biochemical characteristics of fruits of spontaneous and induced mutants enables to single out some high sacchariferous forms.

Table 1. Changes of chemical composition of mutant forms of *Citrus nobilis* Kovano vasse

N	Acidity, mg/ %	vitamin C mg/%	sugars	
			monose %	sucrose %
1087	0.9	43.2	4.3	4,3
1092	1.1	32.1	3,0	5,0
1100	1.0	43.2	3,7	5,1
1556	0.9	34.2	4,7	4,.1
1603	0.9	33.7	3,2	4,8
1612	1.1	40.7	3,3	4,9
1310	1.2	32.7	2,1	6,5
1080	0.9	33.7	2,6	5,6
53	0.9	32.2	3,0	5,0
1312	1.0	32.6	2,2	5,8
1269	0.9	34.3	2,3	5,9
1884	1.0	32.1	3,2	5,0
1504	0.9	33.7	3,2	4,8
control	1.1	38.5	3,6	4,4

Daily dynamics of mutagenic effect of extract received from leaves of sterile forms was studied on onion (*Allium fistulosum*) meristem cells. The data obtained show that 10% water solution concentration of extract makes high mutagenic effect. At its effect on onion primordial roots significant increase of mitotic index (35%±11) as compared with control (5.4%±0.4) is noted. It achieves maximal value after 30 hours of root treatment. At the next periods this index is reduced naturally (13.9±0.7). At the effect of extract ratio of cells being in segmentation phase is changes significantly. Among the phases of mitosis telophase takes the big share (22.3%), and metaphase – the least share (Table 2). The reason of this fact is uncounted in the meantime.

Table 2. Effect of leaf extract of *Citrus nobilis* Kovano vasse sterile forms on onion mitotic index.

Extract concen-tration %	Duration of fixation (h)	Analyzed cell number	Mitotic index %	td*	Number of cells being in mitosis phase, %			
					prophase	metaphase	anaphase	telophase
10	30	1765	35.0±11	23.0	7.5	1.4	3.6	22.3
	60	1068	19.3±1.2	9.1	2.2	0.6	4.8	11.6
	90	2039	13.9±0.7	11.8	3.6	1.3	3.2	5.6
20	30	2234	13.7±0.7	7.3	3.3	1.0	2.5	6.8
	60	2107	6.9±0.5	1.5	2.6	0.6	2.1	1.5
	90	2009	5.2±0.4	1.4	1.5	0.7	1.4	1.4
control	30	2024	7.4±0.5	-	3.6	0.8	1.8	1.0
	60	1993	5.8±0.5	-	1.6	0.8	1.8	1.3
	90	2104	4.,4±0.4	-	1.5	0.9	1.7	1.2

*td - reliability between tested and control variants

Table 3. Mutagenic effect of leaf extract of *Citrus nobilis* Kovano vasse mutant forms.

Extract concentration %	Duration of fixation (h)	Analyzed cell number	Chromosome aberrations	Chromosome aberrations (%) in ana- and telophases					
				td	Bridges	Paired fragment	lagard	Many micronu-cleus	Other disorders
10	30	1765	15.0±0.8	13.8	8.6	8.6	2.7	10.1	69.5
	60	1068	10.0±0.9	9.45	2.7	29.7	8.7	18.9	37.5
	90	2039	13.0±0.7	17.9	2.3	16.6	26.1	11.9	42.8
20	30	2234	10.5±0.6	10.8	11.3	29.5	18.1	4.5	36.3
	60	2107	13.8±0.7	17.36	-	5.1	18.6	9.3	20.9
	90	2009	3.8±0.4	6.36	-	60.0	8.0	-	32.0
control	30	2024	3.2±0.3	-	1.1	33.3	22.2	-	33.3
	60	1993	1.3±0.2	-	33.3	33.3	-	-	33.3
	90	2104	1.0±0.2	-	50.0		-	-	-

It should be also noted that at the extract effect frequency of chromosomal aberrations compared with control is increased significantly in root meristem cells and 30 hours after fixation it reaches the highest value (15.0%±0.7). In control variant frequency of chromosomal aberrations is relatively low (3.2%±0.3). But after extract effect spectrum of chromosomal aberrations becomes diverse (Table 3).

Rather different data are obtained at the effect of 20% extract. In this case mitotic index varies within 5-13% (Table 3). The highest indices of frequencies of chromosomal aberrations are registered 60 hours after fixation. In this case the bridges are mainly observed in control variant, while fragments prevailed in test object (60%) (Table 3).

On the basis of analysis of obtained data we can consider that leaf extract of mandarin have mutagenic feature. Mitotic index, as well as frequency of chromosomal aberrations varies at the effect of extract on the outgrowths depending on concentration. At the effect of mandarin leaf

extract solution of various concentration on onion meristem cells increase of frequency of chromosomal aberrations (bridges, fragments) and mitotic stimulation are observed, which indicates the genetic activity of bioproducts accumulated in plant leaves [Diasamidze, 1995].

Obtained data indicate that selection of mutants should be conducted not only by morphological characteristics. Detection of new mutants according to deviations should be concerned as one of a criteria of study of the level of changes in mutants' growth and development process and results of such studies should be the basis for estimation of perspective forms.

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მანდარინ კოვანო ვასეს (***Citrus nobilis*** **Kovano vasse**) სპონტანური და ინდუცირებული მუტანტების თავისებურებები

ქორიძე მ., ხუხუნაიშვილი რ., დოლიძე ქ., ჯაყელი ე.

შოთა რუსთაველის ბათუმის სახელმწიფო უნივერსიტეტი

(მიღებულია 08.01.2000)

რეზიუმე

მანდარინ კოვანო ვასეს როგორც სპონტანური, ასევე ინდუცირებული მუტაციისას წარმოიქმნება ფენოლოგიური და გენერაციული ხასიათის ცვალებადობანი. ეს ეხება როგორც ზრდის სიძლიერეს, ფოთლის ფორმასა და ყუნწის მორფოლოგიას, ასევე ნაყოფის ფორმასა და შეფერილობას, ეკლიანობას, თესლიანობასა, C ვიტამინის და შაქრების შემცველობას, ადრემწიფადობასა და სხვა ნიშნებს. მათში დაგროვილი ბიოპროდუქტები უჯრედის გენეტიკურ აპარატზე გარკვეული მუტაგენური მოქმედების უნარის მქონეა, რაც, თავის მხრივ, ზრდის თვით მანდარინების სპონტანური მუტაციების აღმოცენების შესაძლებლობებს.

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ENGAGEMENT OF DIFFERENT Fcγ RECEPTORS IN PHAGOCYTOSIS OF IMMUNE COMPLEXES CONTAINING ANTIBODIES TO MUTATED HUMAN CHORIONIC GONADOTROPIN β CHAIN (hCG β) AND NATIVE hCG MOLECULE

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Abstract

The aim of this study was to investigate the role of Fcγ receprors (FcγR) *in vitro,* in phagocytosis of hormone-antibody complexes formed after immunization with the mutant hCGβ(R68E). Native, FITC labeled hCG was added to the sera from mutant hCGβ(R68E) immunized rabbits. The suspensions of phagocytic cells were selectively incubated with monoclonal antibodies to FcγRI, FcγRII, FcγRIII and the complement receptor 3 in order to block their functions and phagocytosis was visualized by flow cytometry. We show that the phagocytosis of immune complexes containing anti-hCGβ(R68E) sera of rabbits and native hCG molecules by monocytes and neutrophils is mediated by FcγRI(CD64) and FcγRIII(CD16) respectively in cooperation with the complement receptor 3 (CR3).

Key words: Fcγ receprors, human chorionic gonadotropin β chain (hCG β), phagocytic activity

Introduction

Pregnancy-related hormone Human chorionic gonadotropin (hCG), which is a member of the glycoprotein hormone family, is normally produced during pregnancy by early blastocysts and later by trofoblastic cells of placenta [Stenman et al., 2006]. HCG is a heterodimeric molecule and consists of an α-chain which is shared with the other members of glycoprotein hormone family follicle-stimulating hormone (FHS), thyroid-stimulating hormone (TSH) and luteinizing hormone (hLH)) and hormone-specific β-chain [Stenman et al., 2006]. It is responsible for the formation of corpus luteum thus promoting a production of progesterone and oestrogen [Stenman et al., 2006; Delves et al., 2002]. Hence a vaccine based on hCG has been proposed as a mean to control fertility [Stenman et al., 2006; Delves et al., 2002; Naz et al., 2005] and various vaccine formulations based on hCG have been developed for this purpose.

Besides hCG is a tumor associated antigen over expressed in a variety of common cancers, including those of the colon, lung, pancreas, esophagus, breast, bladder, stomach, prostate, cervix

and ovary. Unlike pregnancy and in trophoblastic tumors, where it is the holo-hormone that is expressed, in nontrophoblastic tumors the hCG ß-chain is most often expressed in the absence of α-chain [Shzh et al., 2008]. The ectopic expression of this hormone may play a pathogenic role in cancer patients. Clinical studies have showed that the anti-hCG response plays an important role in life-saving of patients with these tumors. Also, inhibitory effects of anti-hCG antibodies on the growth of tumor cells have been demonstrated on animal models. Therefore, hCGβ has been considered as a candidate target protein for vaccine development aimed for active cancer immunotherapy [Shzh et al., 2008; Delves et al., 2007].

Vaccines, which incorporate the full-length hCGβ-chain, stimulate the production of antibodies that cross-react with hLH due to the 85% amino acid sequence homology of the first 110 amino acids of the β-chains of the two hormones. As a strategy aimed at reducing hLH crossreactivity of hCG several hCGβ-chain mutants were engineered by using oligonucleotide-directed site-specific mutagenesis at University College London, UK [Delves et al., 2007; Porakishvili et al., 2002]. One of the mutant molecules hCGβ(R68E) containing an arginine to glutamic acid replacement at a position 68 in the protein sequence has grossly diminished ability to provoke hLH cross-reactive antibodies whether administered intranasally to mice or intramusculary to rabbits [Delves et al., 2007; Porakishvili et al., 2002]. Much of anti-hCGβ(R68E) antibodies were re-focused towards normally weakly immunogenic C-terminal peptide (CTP) unique for hCGβ and not shared by hLH [Porakishvili et al., 2002]. In the mutant hCG molecule CTP acquires the properties of an immunodominant epitope [Porakishvili et al., 2002]. It was essential to demonstrate that possible interaction between endogenous native hCG with hCG/hLH receptor expressed on the target cells can be prevented following immunization with mutant vaccine prototype. We have previously shown that the immune complexes (IC) formed by antibodies raised against mutated hCGβ(R68E) in rabbit sera bound to labeled native human hCG in vitro and are taken up and internalised by monocytes and neutrophils [Chikadze et al., 2003].

The aim of this study was to investigate the role of different Fcγ-receptors in phagocytosis of the immune complexes containing native hCG and anti-hCGβ(R68E) antibodies.

Materials and Methods

Donors: The research was carried out at the Department of Immunology Javakhishvili Tbilisi State University. We have examined the peripheral blood of 40 healthy female donors aged 20-50.

Antigens: In our experiments we used the following antigens: **Native hCGαβ** – hCGαβ-heterodimer purified from human pregnancy urine (Zimed, USA); **Native hCGβ** – hCGβ-chain purified from human pregnancy urine (Zimed, USA); **Mutant hCGβ(R68E)** – recombinant hCGβ comtaining a single amino acid substitution expressed in High Five™ cell cultures (Invitrogen, USA) using baculovirus expression system. Recombinant protein was purified from supernatants of High Five cell cultures using Immobilized metal affinity chromatography (IMAC). Supernatants were loaded on HiTrap Chelating HP 1ml columns (Pharmacia), charged with Ni ions and His-tagged proteins eluted with Imidazole step gradient (0.04-0.4M). Purity was examined by SDS PAGE on Phast Gel system (Pharmacia) followed by Silver staining or Western Blotting procedure (Fig.1).

FITC-conjugation: The hCG (Sigma) solution of 1 mg/ml concentration preserved in carbonate-bicarbonate buffer (CBB) was prepared for FITC labelling. FITC (Sigma) was dissolved separately at 0.5 mg/ml in CBB. 200 µl of the hCG solution was placed in a test-tube and 50 µl of the FITC solution was added drop-by-drop (the amount of the FITC designated for adding had been selected according to the hCG molecular weight and manufacturer's recommendations). After

incubation for 2 hrs at RT, the FITC-labelled protein was cleared of the unconjugated fluorescene through Sephadex G-25M column. The conjugated protein was preserved at 4^0C in an impermeable tube. The binding of antibodies with FITC-conjugated hCG was tested by indirect ELISA.

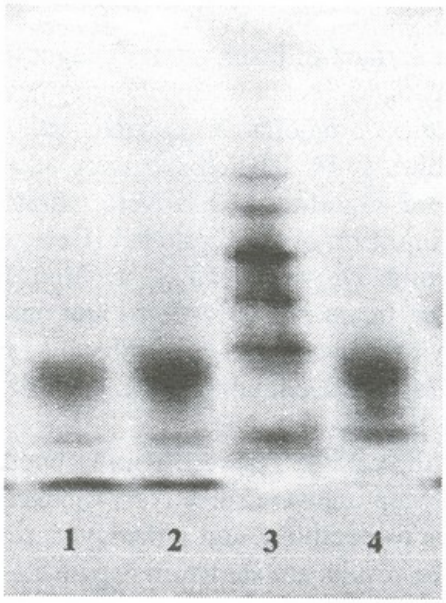

Fig.1. SDS PAGE analysis of purified R68E samples on Phast System Using PhastGel 12.5% homogeneous, followed by Western Blotting. Lane 1, 2, 4 R68E purified from High Five cell supernatants. Lane 3- molecular weight markers- 150, 100, 75, 50, 35, 15kda.

Formation of the immune complexes: FITC-conjugated hCG at final concentration 3μg/ml and the mutant sera at a dilution 1:25 in phosphate-buffered saline (PBS, Sigma) were incubated at 37°C for 2 hours and then at 4°C for 24 hours for IC precipitation. The optimal conditions for IC phagositosis by monocytes and neutophils were established in our previous study [Chikadze et al., 2003]. The control samples contained hCG molecules only with PBS instead of antibodies. All samples were taken in duplicates.

Separation of mononuclear cells and neutrophiles from peripheral blood: 10 ml of peripherial blood was diluted 1:1 with Hanks' Balanced Salt Solution (HBSS, without Ca^{2+}/Mg^{2+} and phenol red, Gibco BRL)) and carefully layered on the 3 ml of the Histopaque 1119 (density 1.119g/cm^3, Sigma Diagnostics), and Histopaque 1077 (density 1,077g/cm^3, Sigma Diagnostics). After centrifugation at 800g for 40 min, the interphases of PBMC and PMN were separated and collected in different tubes, each sample was washed in HBSS at 400g for 10 min, resuspended in 1ml of medium RPMI 1640 (Sigma), counted in Haemacytometer and concentration adjusted at 1x 10^6/ml.

Blocking of Fcγ-receptors (FcγR) and complement receptors (CR) using monoclonal antibodies: The suspentions of phagocytic cells were incubated with monoclonal antibodies - mAbs (anti-CD64 (FcγRI) (Ancell); anti-CD32 (FcγRII) (Ancell); anti-CD16 (FcγRIII) (Sorbent); anti-CD11b (CRIII) (Sorbent)) at 4°C for 30 minutes for blocking the receptors on the surface of the cells.

Phagocytosis of the immune complexes: The mixture of ICs or the control preparation and cells were incubated at 37°C for an hour in test-tubes. The percentages of FITC-positive phagocytic cells were measured by flow cytometry (FACScan, Becton & Dickinson) by gating on monocytic and PMN populations.

Statistical analysis was performed by the Student t-test. The values on the charts represent average (m) and standard deviation (SD).

Results and Discussion

Ab/Ag immune complexes may initiate phagocytosis via interaction of Fc region of IgG antibodies with different Fcγ receptors on phagocyting cells. The three classes of Fcγ receptors, FcγRI (CD64; a 72-kDa sialoglycoprotein), FcγRII (CD32; a 40-kDa sialoglycoprotein) and FcγRIII (CD16) are found on the surface of professional phagocytes (Table 1) [Shah et al., 2008]. We have investigated the role of these receptors in phagocytosis of the ICs formed in vitro by interaction of antibodies from sera of rabbits immunized with hCGβ(R68E)-TT conjugate and FITC labeled native hCG molecules. FITC labeled ICs were added to monocytes and neutrophils isolated from health volunteers and engulfing of ICs were monitored by flow cytometry. Initially we have examined the degree of injestion of FITC labeled ICs by measuring possible quenching of external signal with non-permeating dye such as Trypan Blue. No differences were detected in phagocytic activity in Trypan Blue treated (60.5+10.8%) and untreated (60.7+12.1%) monocytes. Similar results were obtained with neutrophils- 52.5+8% in Trypan Blue treated samples and 55.5+10.9% in untreated ones. Thus hCG containing ICs are fully internalized by monocytes and neutrophils [Chikadze et al., 2003].

We further investigated the role of Fcγ receptors in engulfing of hCG containing labeled ICs. FcγRI, FcγRII and FcγRIII receptors were selectively blocked with anti-CD64, anti-CD32 and anti-CD16 monoclonal antibodies respectively and phagocytic activity measured before and after blocking the receptors. (Fig.2). The results are shown in Figure 2.

Table 1. Distribution and properties of different Fcγ receptors

Receptor	FcγRI (CD64)	FcγRII-A (CD32)	FcγRII-B2 (CD32)	FcγRII-B1 (CD32)	FcγRIII (CD16)
MW	72kDa	40kDa	40kDa	40kDa	50-70kDa
Binding Affinity	IgG1 10^8M^{-1}	IgG1 $2x10^6M^{-1}$	IgG1 $2x10^6M^{-1}$	IgG1 $2x10^6M^{-1}$	IgG1 $5x10^5M^{-1}$
Cell Type	Macrophages Neutrophils Eusinophils Dendritic cells	Macrophages Neutrophils Eusinophils Platelets Langerhans cells	Macrophages Neutrophils Eusinophils	B cells Mast cells	NK cells Eusinophils Macrophages Neutrophils Mast cells
Function	Uptake Induction of killing	Uptake Granule release	Uptake Inhibition of stimulation	No uptake Inhibition of stimulation	Induction of killing

FcγRI (CD64) is a high affinity receptor for monomeric IgG. CD64 plays an important role in clearance of immune complexes and in antibody dependent cytotoxicity [Delves et al., 2007]. We have revealed significant difference in engulfing activity of phagocytic cells after adding anti-CD64 mAb to cell suspensions. Our data shows that the percentages of FITC-positive monocytes, when cells are pre-treated with anti-CD64 antibodies, are significantly decreased (19.9±8.6%) compared with untreated samples (57±3.4%) (Fig. 1a). These results indicate that FcγRI(CD64) is involved in mediating phagocytosis by monocytes in our system, which coincides with established view that FcγRI(CD64) mediated uptake is essential for the clearance of ICs [Vugt et al., 1999].

FcγRII (CD32) molecule is a low affinity receptor for immune complexed IgG and has signal-transducing capabilities involved with humoral and cell-mediated immune responses [Schlossman et al., 1995]. FcγRIIA (activating receptor) is found mainly on phagocytic cells (neutrophils, monocytes,and macrophages), whereas FcgRIIB (inhibitory receptor) is expressed in B lymphocytes and mast cells [Fridman, 1991]. There was no significant difference in phagocytic

activity, measured as the percentages of FITC-positive cells, between the samples pre-incubated with anti-CD32 mAb (39±5.8% -monocytes, 42±8.7%-neutrophils) and untreated ones(42,3±4.5% -monocytes, 44.5±3.8%-neutrophils) (Fig. 1 b). Therefore, FcγRII does not take part in phagocytosis of labeled hCG containing ICs.

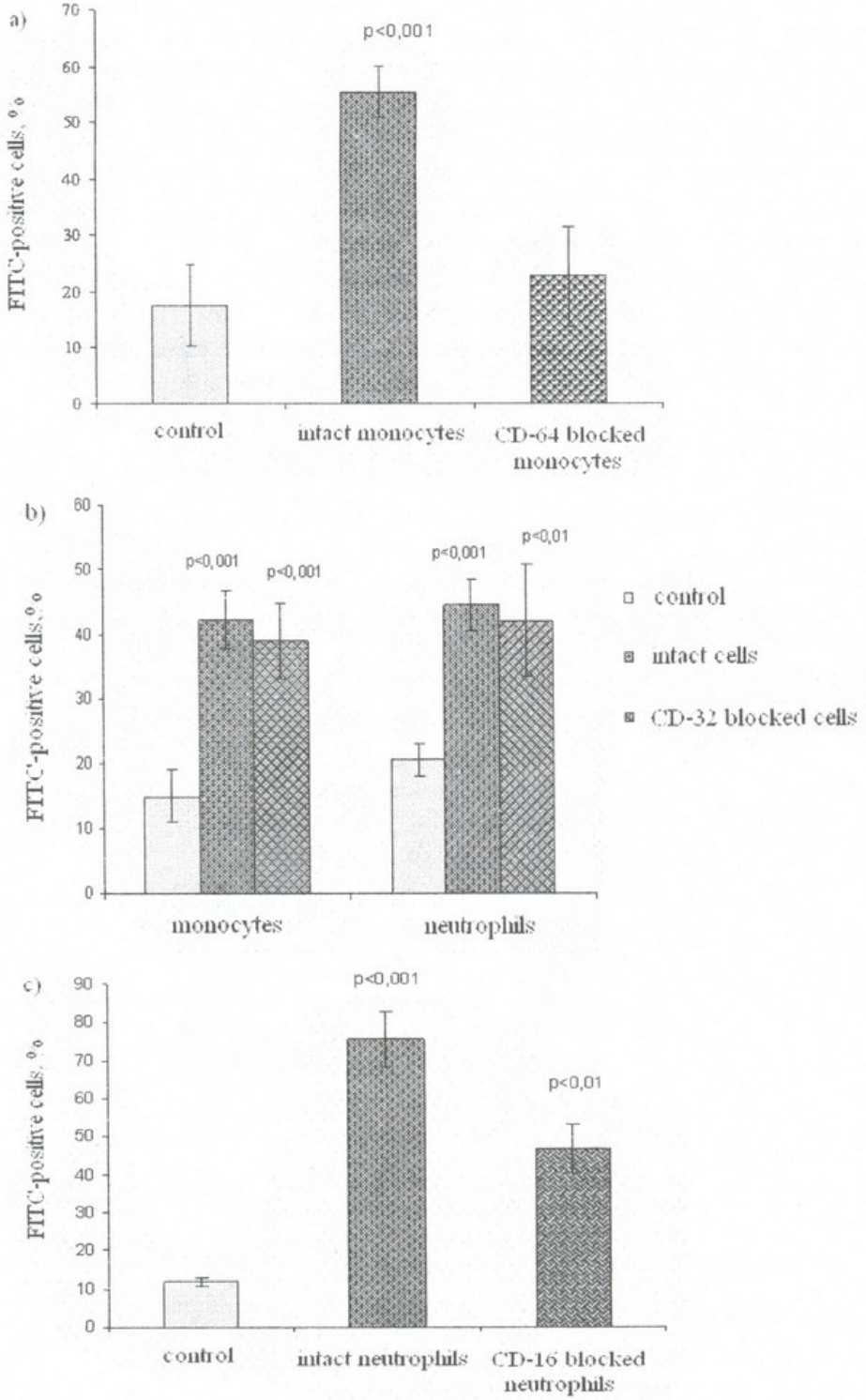

Fig. 2. Engulfing activity of FITC labeled ICs by monocytes and neutrophils before and after selectively bloking FcγRI, FcγRII and FcγRIII. Monocytes and neutrophils were isolated from healthy female volunteers. Native hCG was labelled with FITC and allowed to interact with antibodies from the sera of immunized rabbits. Cells were incubated with a) CD16 (anti-FcγRI), b) CD32(anti-FcγRII), c) CD16 (anti-FcγRIII) monoclonal antibodies at 4^0 for 30 min. FITC positive cells counted by Flow Cytometry.

On the contrary, when neutrophils were incubated with anti-CD16 antibodies this caused decrease in their phagocytic activity (47±6.3% compared to 75±7.2% of untreated ones). FcγRIII (CD16) is encoded with two identical genes FcγRIIIA and FcγRIIIB. A single nucleotide change leads to a difference (substitution of the Phe at amino acid 185 in FcγRIIIA with Ser in FcγRIIIB) that creates a signal for addition of GPI anchor, rather than a transmembrane domain in FcγRIIIB. It is FcγRIIIB that is constituently expressed on neutrophils, while macrophages, some monocytes, natural killer cells and some T cells express FcγRIIIA. FcγRIIIB binds monomeric IgG very poorly, but may function in cooperation with other receptors such as CR3, which belongs to phagocyte specific integrins of β_2 family and acts as a receptor for iC3b fragment of C3. This major adhesion molecule is formed by α-chain (CD11b) noncovalently linked to β-chain (Mac-1, CD18) [Vossebeld et al., 1997; Jongstra-Bilen et al., 2003]. CR3 expression is required for FcγRIIIB receptors to interact with phagocyte cytoskeleton for adhesion and injestion, which was proved in transfection experiments [Krauss et al., 1994]. A close lateral association of those two receptors has been suggested also by demonstration of resonance energy transfer [Poo et al., 1995]. To investigate involvement of CR3 in phagocytosis of labeled ICs we have incubated phagocytic cells in our experiments with anti-CD11b antibodies and measured engulfing activity consequently. Pretreatment of monocytes and neutrophils had indeed decreased significantly FITC positive cell counts, indicating CR3 receptor involvement in this process. The percentages of FITC-positive intact monocytes and neutrophils were 61±6.8% and 72.3±10.2% respectively, whereas the percentages of FITC-positive anti-CD11b pre-treated monocytes and nutrophils were 34±7.6% and 38.9±4.9% (Figure 3).

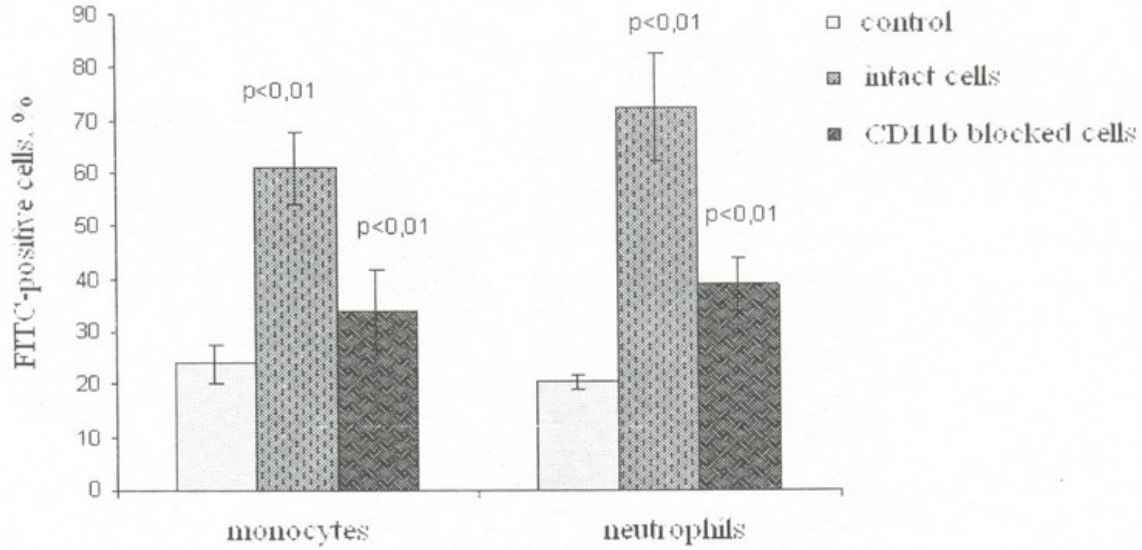

Fig. 3. CR3 receptor envolvement in phagocytic activity of human monocytes and neutrophils. Monocytes and neutrophils were isolated from healthy female volunteers. Native hCG was labelled with FITC and allowed to interact with antibodies from the sera of immunized rabbits. Cells were incubated with anti-CD11b (anti-CR3) monoclonal antibodies at 4^0 for 30 min. FITC positive cells were counted by Flow Cytometry.

Our data supports the notion that the role of CR3 in phagocyte effector mechanism extends beyond its function as a complement receptor and it may be essential component of the adhesive and phagocytic functions of phagocyte plasma membrane receptors by promoting efficient interaction between the actin cytoskeleton and phagosome plasma membrane.

In conclusion, we have demonstrated involvement of FcγRI, FcγRIII and CR3 in phagocytosis of ICs formed in vitro by interaction of FITC labeled hCG and anti-hCG antibodies in the sera from rabbits immunized with BAC hCGß(R68)-TT conjugate. This points to the possibility

that hCG containing immune complexes formed in vivo could be successfully cleared by phagocytosis and this could in turn inhibit tumor growth promoting functions of hCG molecule. Altogether, our data argues in favor of effectiveness of BAChCGß-TT formulation for use as anti-cancer vaccine.

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სხვადასხვა Fcγ რეცეპტორების ჩართულობა ადამიანის ქორიონული გონადოტროპინის მუტანტური β-ჯაჭვის და ნატიური ქორიონული გონადოტროპინის შემცველი იმუნური კომპლექსების ფაგოციტოზში

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რეზიუმე

ჩვენს წინა ნაშრომებში ნაჩვენებია, რომ როდესაც ადამიანის ქორიონული გონადოტროპინის (აქგ) მუტანტური β-ჯაჭვი, რომელშიც 68-ე ამინომჟავა არგინინი ჩანაცვლებულია გლუტამინის მჟავით [აქგβ(R68E)], კონუგირებულია ტეტანუსის ტოქსოიდთან გენერირებს ძლიერ ანტი-აქგ სპეციფიკურ პასუხს ბოცვრებში. ეს ანტისხეულები კარგად უკავშირდებიან ნატიური მოლეკულის აქგβ C-ტერმინალურ დაბოლოვებას და ქმნიან იმუნურ კომპლექსებს (იკ), რომლებიც სირთულის გარეშე ფაგოციტირდებიან *in vitro* მონოციტებისა და ნეიტროფილების მიერ.

მოცემული კვლევის მიზანია Fcγ რეცეპტორების (FcγR) როლის შესწავლა მუტანტური მოლეკულა აქგβ(R68E)-ით იმუნიზაციის შედეგად წარმოქმნილი ჰორმონი-ანტისხეული იკ-ების ფაგოციტოზში *in vitro*. ამისათვის, აქგβ(R68E)-ით იმუნიზებულ ბოცვერთა შრატებს ვუმატებდით ნატიურ, ფლუორესცინ იზოთიოციანიტით (ფითც) მონიშნულ აქგ-ს. ფაგოციტურ უჯრედებს კი შერჩევითად ვაინკუბირებდით ანტი-FcγRI, ანტი-FcγRII, ანტი-FcγRIII და ანტი-კომპლემენტის რეცეპტორი 3 (CR3) მონოკლონურ ანტისხეულებთან აღნიშნული რეცეპტორების ბლოკირებისათვის. საბოლოოდ, ფაგოციტოზს ვაკვირდებოდით გამდინარე ციტომეტრით. მიღებული შედეგებიდან ჩანს, რომ ბოცვრების ანტი-აქგβ(R68E) შრატებით და ნატიური აქგ მოლეკულებით შექმნილი იკ-ების ფაგოციტოზი მონოციტებისა და ნეიტროფილების მიერ გაშუალებულია FcγRI(CD64) და FcγRIII(CD16) რეცეპტორებით და კომპლემენტის რეცეპტორი 3 ამ პროცესის თანამონაწილეა.

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CHARACTERISTICS OF DISTRIBUTION OF SOME ERYTHROCYTIC GROUP ABO, RH, KELL, MN SYSTEM ANTIGENS AND ALLELES AMONG PULMONARY TUBERCULOUS PATIENTS

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Abstract

Relation of erythrocytic group antigen composition with pulmonary tuberculosis was studied. Immunoserological methods with test-systems of anti -AB, -B, -A, -D, -CD(G), -C, -c, -E, -Ce, -e, -K, -M, -N specificities, standard erythrocytes of O(I), A(II), B(III) groups and serum were used. Obtained data were treated statistically. It was revealed that O(I) and B(III) group carriers are reliably sensitive to pulmonary tuberculosis, but A(II) group carriers less sensitive. High concentration of p(M) allele was recorded in tubercular patients, and vice versa, concentration of q(N) allele is higher (0.35) in control group. We might say that carrying of N-allele is somehow "defensive" feature. High distribution frequency of D-antigen was noted in patients. Reliable high frequency of CC was registered in patients, whereas distribution frequency of Cc and cc is higher in control group. Majority of consumptive patients (72.0±4.8%) is M-antigen carrier, which is explained by sensitivity of this antigen to tuberculosis, while the portion of N phenotypic groups is relatively higher in control group (21.0±4.07%).

Key words: pulmonary tuberculosis, group antigens, immunoserological methods

Introduction

Blood erythrocytic group antigens are genetically stiff determinate characteristics [Instee, 1990]. They have complex biological and medical significance.

The main value of erythrocytic group antigens is related with immune features of organism. They are especially important in transfusiology [Schonewille et al., 2006, epidemiology [Cartron, 1994; Shubin, 1997] and transplantology [Bolan et al., 2001; Bucin et al., 2006]. Significance of those systems while study of human genetics [Cartron, 1994; Shubin, 1997] and especially of its population peculiarities [Kucher et al., 2000; Nersisian et al., 1996] should be noted.

Associativity of blood group antigens with various pathologies is nonuniform [Platonova, 1999; Su M et al., 2001; You WC et al., 2000; Umit Tursen MD et al., 2005].

In the viewpoint of association of ABO system antigens with tuberculosis alternative results are recorded in scientific literature [Reddy, Usha, 1990; Volkova et al., 1993].

As all alloantigens present serologically recorded product of the corresponding gene, study of correlation of erythrocytic antigen composition with different pathologies is possible with rather high accuracy. We aimed to establish correlation of erythrocytic group antigens with pulmonary tuberculosis. Such researches enable us to find out an expression of which group antigens is associated with this disease. Study of distribution frequency of erythrocytic antigens in patients and their comparison with control group make possible to single out individuals susceptible to tuberculosis with "high risk" and to carry out preventive measures to avoid this illness.

Materials and Methods

We used immunoserological methods with test-systems of the following specificities: anti -AB, -B, -A, -D, -CD(G), -C, -c, -E, -Ce, -e, -K, -M, -N (OOO "Gemostandard", Moscow), standard erythrocytes of O(I), A(II), B(III) group and serum. Obtained data were treated statistically.

Distribution frequency of ABO system genes was calculated by F. Bernstein formula, which is used for study of three-allelic genetic systems (frequencies of O, A and B genes are expressed by *r, p* and *q* letters):

$$r = \sqrt{O};\ p = 1 - \sqrt{A+O};\ q = 1 - \sqrt{B+O},$$

where *O, A* and *B* is the ratio of the number of carriers of O(I), A(II) and B(III) groups to the total number of researched individuals.

Frequencies of Rh-system antigens and haplotypes were calculated using the following formulas:

$$D = 1 - \sqrt{dd};\ C = 1 - \sqrt{cc};\ E = 1 - \sqrt{ee};\ c = 1 - \sqrt{CC};\ e = 1 - \sqrt{EE},$$

where *D, C, E, c, e* - is the ratio of the number of those gene carriers to the total number of researched individuals; *dd, cc, ee, CC* and *EE* – frequencies of the corresponding phenotypes.

Frequencies of Rh haplotypes were evaluated by A.E.Mourant formulas:

$$cde = \sqrt{ccddee};\ Cde = \frac{Ccddee}{2cde};\ cdE = \frac{ccddEe}{2cde};\ cDe = \frac{ccDee}{2cde};$$

$$cDE = \sqrt{ccDEE + cdE^2} - cdE;\ CDe = \sqrt{CCDee + Cde^2} - Cde;$$

$$CDE = \frac{CCDEe}{2(CDe + cde)},$$

where *ccddee, Ccddee, ccddEe, ccDee, CCDee* and *ccDEE* are the frequencies of the corresponding phenotype.

Concentration of RhD and Kell system alleles was determined by means of the formulas:

$$q = \sqrt{\frac{n_{aa}}{N}};\qquad p = 1-q,$$

where n_{aa} is the recessive homozygotes (dd and kk) according to mentioned locuses; N – total number of researched individuals.

To establish concentration of MN system alleles the following formulas were used:

$$P = \frac{n_A + \frac{1}{2} n_{AB}}{N}; \qquad q = \frac{n_B + \frac{1}{2} n_{AB}}{N}$$

where n_A is the number of M-phenotype carriers, n_{AB} – MN phenotype carriers and n_B – N phenotype carriers.

Dispersion of antigen and gene frequencies was calculated by the formula: $M = \sqrt{P(100-P)/n}$, where P – antigen frequency (%), n – number of researched individuals.

Results and Disscusion

Obtained results show that O(I) and B(III) group carriers are reliably sensitive to pulmonary tuberculosis (PT). But A(II) group carriers are less vulnerable, they are somehow characterized by immune stability (Fig. 1). Our data are in accordance with the data existed in scientific literature [Platonova, 1997].

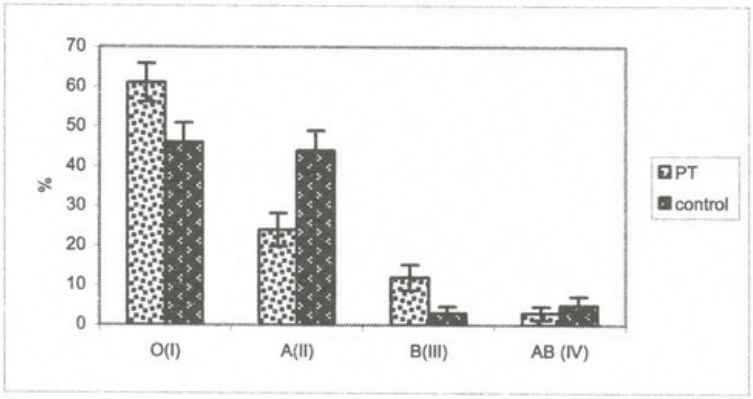

Fig.1. Features of ABO phenotype distribution among PT patients and control group

Among PT patients reliable high concentration of r-allele compared with the control group is noted. Within the PT patients p-allele concentration is also higher insignificantly. Concentration of q-allele in control group is 2.14 times higher.

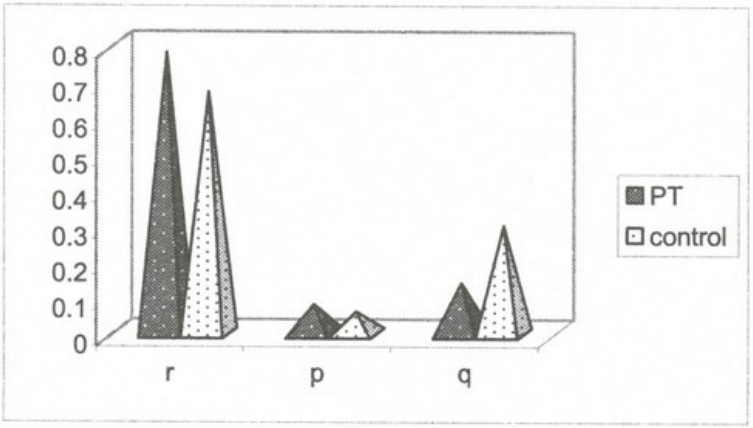

Fig. 2. Concentration of *r*, *p* and *q* alleles among PT patients and control group

Within PT diseased group high distribution frequency of D-antigen was recorded (Fig. 3). We can say that D-antigen correlates with tuberculosis and thus concentration of D allele is reliably

high in diseased group. It means that in carriers of this allele (individuals, on which erythrocyte surface D antigen is expressed) theoretical probability of PT expression is high. Among those patients tuberculosis is detected 1.05 times more than among d-allele carriers.

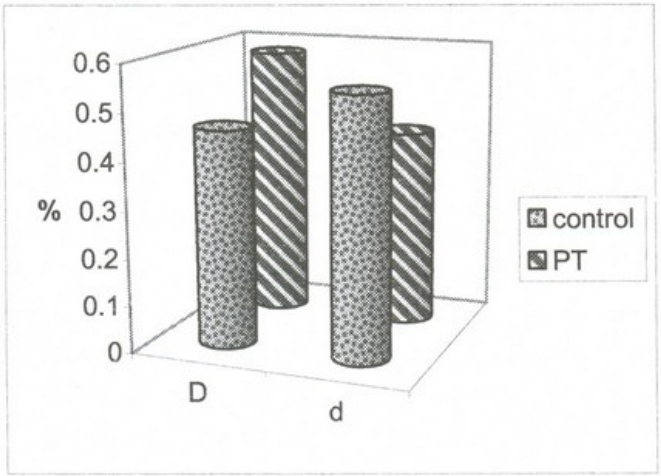

Fig. 3. Concentrations of D and d alleles within PT and control groups

It was noted that antibacterial antibody titre is considerably low in D positive individuals [Donskov, 2001]. Intensity of immune response caused by some bacterial anatoxins is correlated with D and E antigens.

CC genotype is presented by reliably high frequency, while distribution frequency of Cc and cc variants among control group is higher (fig. 4).

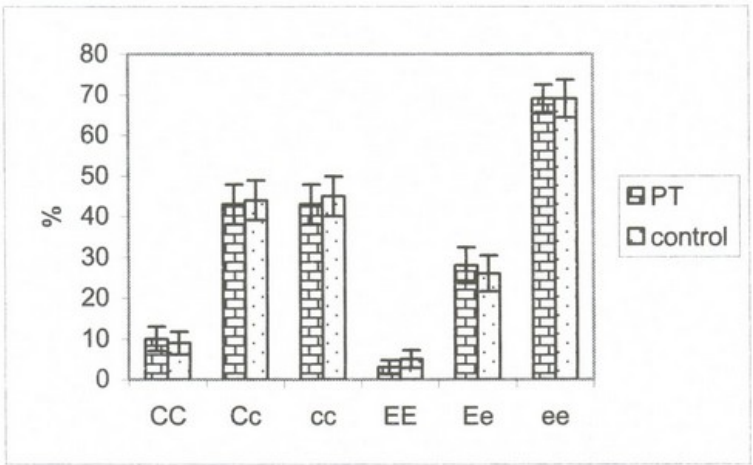

Fig. 4. CC, Cc, cc, EE, Ee, ee genotype frequencies among PT and control groups

Positive correlation was registered between Ee genotype and PT. **ee** genotype is presented with equal percentages among diseased and control groups. Carriers of EE genotype appeared more stable against PT.

Within the population of PT patients concentration of **cde** haplotype equals to 0.41, while in control group it equals to 0.36. CDE haplotype concentration in PT patients' group is 0.01. This haplotype was not educed in the control group and evidently it is associated with the disease. But concentrations of cDe and cDE haplotypes is higher in control group (Fig. 5), which confirms that

carriers of those haplotypes are less susceptible to tuberculosis and characterized with immune stability.

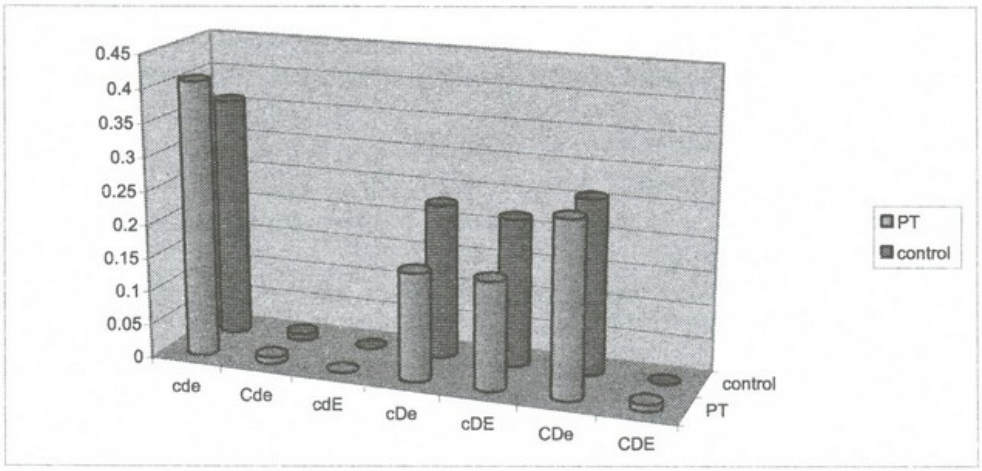

Fig. 5. Concentration of Rh haplotypes among PT and control groups

Certain relations were recorded among diseased and control groups according to distribution features of MN group phenotypes. Majority of PT patients (72.0±4.8%) is M antigen carrier, which is explained by sensitivity of this antigen against tuberculosis. On the other hand, specific content of N phenotype groups is relatively higher (21.0±4.07%) in control group. In the chemical viewpoint MN system antigens belong to glycophorin-A. Cyaloglycoprotein, being the basic ligand for some bacteria and viruses, is identified by acetylneuraminic acid, which is a composite of glycophorin-A. It was established that genetic variability of MN system may affect the bacterial and viral competition [Bottini et al., 2005]. By statistical methods concentrations of p(M) and q(N) alleles among diseased and control groups were calculated. It was shown that p(M) allele concentration in PT patients group was reliably higher (0.77) compared with control group (0.66). And visa versa, q(N) allele concentration among control group is higher (0.35) (Fig. 7).

We can say that carrying of N allele is somehow "protective" feature. Risk of disease development is 1.5 times lower among this allele, and accordingly antigen carriers.

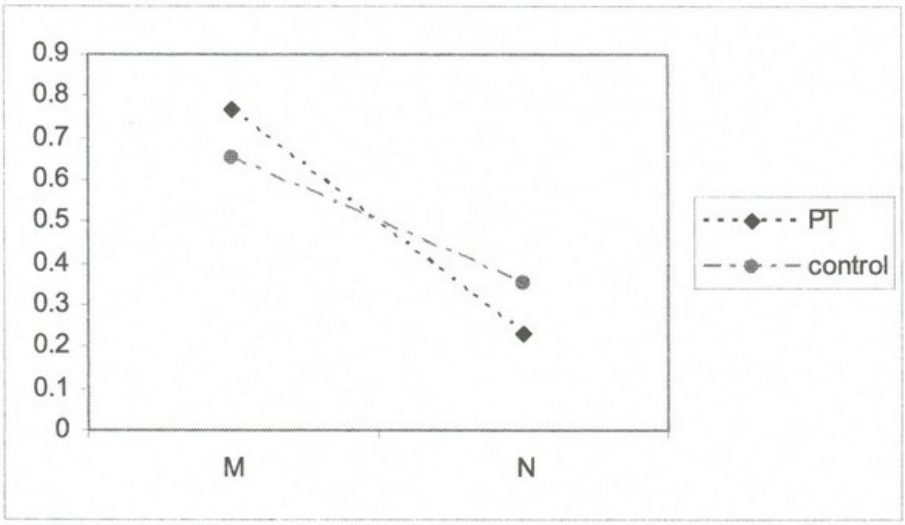

Fig. 7. Concentration of M and N alleles within PT and control groups

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სისხლის ზოგიერთი ერითროციტული ჯგუფური **AB0, Rh, Kell, MN** სისტემის ანტიგენებისა და ალელების გავრცელების თავისებურებები ფილტვის ტუბერკულოზით დაავადებულ პაციენტებში

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(მიღებულია 25.12.2008)

რეზიუმე

შესწავლილია ერითროციტური ჯგუფური ანტიგენების კორელაციური კავშირები ფილტვის ტუბერკულოზთან (ფტ). გამოყენებული იქნა იმუნოსეროლოგიური მეთოდები შემდეგი სპეციფიკურობის მქონე ტესტ-სისტემებით: ანტი - AB, -B, -A, -D, -CD(G), -C, -c, -E, -Ce, -e, -K, -M, -N, სტანდარტული 0(I), A(II), B(III) ჯგუფის ერითროციტები და შრატები. მასალა დამუშავდა სტატისტიკური მეთოდების გამოყენებით. ნაჩვენებია, რომ 0(I) და B(III) ჯგუფის მტარებლები სარწმუნოდ მგრძნობიარენი არიან ფტ-ს მიმართ. A(II) ჯგუფის მტარებლები შედარებით ნაკლებად ექვემდებარებიან აღნიშნულ დაავადებას. ფტ-ით დაავადებულებში გამოვლინდა p(M) ალელის სარწმუნოდ მაღალი კონცენტრაცია. q(N) ალელის კონცენტრაცია კი პირიქით, დონორებში უფრო მაღალია (0.35). შეიძლება ითქვას, რომ N ალელის მტარებლობა გარკვეულწილად "დამცველობით" ნიშანს წარმოადგენს. დაავადებულთა პოპულაციაში გამოიკვეთა D ანტიგენის გავრცელების მაღალი სიხშირე. დაავადებულებში სარწმუნოდ მაღალი სიხშირითაა წარმოდგენილი CC ვარიანტი, მაშინ, როცა Cc და cc ვარიანტთა გავრცელების სიხშირე დონორებში უფრო მაღალია. ფტ-ით დაავადებულთა უმრავლესობა (72.0±4.8%) M ანტიგენის მტარებელია, რაც აიხსნება აღნიშნული ანტიგენის მგრძნობელობით დაავადების მიმართ. სამაგიეროდ, დონორებში შედარებით მეტია N ფენოტიპურ ჯგუფთა ხვედრითი წილი (21.0±4.07%).

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CORRELATION OF ERYTHROCYTE ABO AND Rh-Hr GROUP ANTIGENS WITH BREAST CANCER

NAKASHIDZE I., NAGERVADZE M., KHUKHUNAISHVILI R., DIASAMIDZE A.

(Received December 15, 2008)

Abstract

The prevalence of ABO, Rh-Hr group antigens in 105 patients with breast cancer and in the same number of healthy donors has been studied. Immunoserological methods have been used to identify the antigens. The studies have demonstrated sensitivity of A (II) and AB (IV) blood group carriers to the aforesaid pathology. A relatively high frequency of D antigen (94.24 ± 2.28%) has fixed in the patients with breast cancer, which exceeds that of in the control group (82±3.8%). The diseased population has also revealed a high occurrence frequency of B antigen.

Key words: breast cancer, erythrocyte, group antigen.

Introduction

The medical literature reports on a correlation between various types of tumor and erythrocyte antigens [Henderson et al., 1993; Lee et al., 1991; Su et al., 2001; You et al., 2000]. Since every alloantigen represents a serologically identified product of a respective gene, study of the correlation between the erythrocyte antigenic composition and pathologies of various types appears to be rather promising and possible with a high accuracy.

It is to be mentioned that breast tumor is rather prevalent in Georgia today. It can be explained by rather complicated socio-economic conditions, on the one hand, as well as by a possible genetic predisposition of such patients to the determinative genetic factors of the erythrocyte group antigens, on the other hand.

The literature data related to the above problems are rather numerous and in some cases, even alternative [Anderson, Haas, 1984; Constantini et al., 1990; Holdsworth et al., 1985]. It should be also noted that such data for our region are not available at all.

Materials and Methods

105 patients with breast cancer were investigated for erythrocyte group antigens. The material was supplied by the Ajara Oncology Center, and the experiment was conducted in the Laboratory of Immonogenetics of Shota Rustaveli State University. 105 healthy donors were used as a screening group.

The study was conducted by employing immunoserological methods The following specific test systems were used: anti -AB, -B, -A, -D, -CD (G), -C, -c, -E, -Ce, -e, -K, -M, -N (GEMOSTANDART LTD, Moscow), standard 0(I), A(II), B(III) group erythrocytes and standard 0(I), A(II), B(III), AB (IV) sera.

Results and Discussions

A relatively high percentage rate of the A (II) and AB (IV) phenotypic group carriers was observed in the patients with breast cancer compared with the donor populations (Fig. 1).

It can be noted that the A (II) and AB (II) blood group carriers are more responsive to mammary breast tumors, while the O (I) and (A (II) blood group carriers are less prone to this pathology.

A comparatively higher D-antigen carrying capacity was fixed in the patients with mammary breast tumor according to the Rh-system antigens, where its occurrence frequency accounts for 94.24±2.28% and somewhat exceeds that of in the donors (82 ± 3.8%).

Interesting correlation was also observed by the E-antigen. The diseased showed high rate of the E-antigen, namely – 40 ± 4.2%, whereas the same indicator in the donors makes 23 ± 4.2% (Fig. 2).

Concentration of D-allele (0.77) is increased in the investigated diseased population as compared with the donor population (0.55) (Fig. 3).

The obtained results show that such studies will enable to identify expression of which group antigen is associated with the abovementioned disease. Investigation of the occurrence frequency of erythrocyte antigens in the patients with breast cancer and their comparison with healthy donors will make it possible to identify 'high risk' group individuals prone to the pathology and to conduct preventive therapeutic measures in order to avoid the disease.

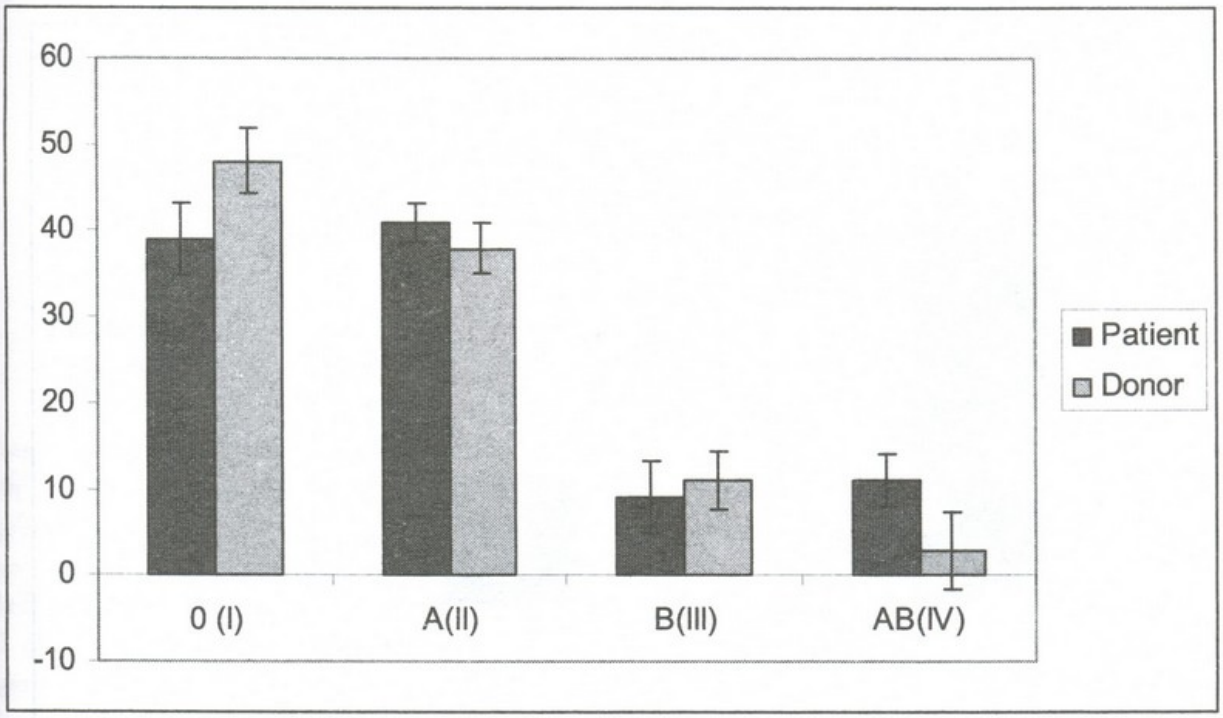

Fig. 1. Features of occurrence frequency of the ABO-system phenotypic groups in the diseased with breast cancer and the healthy donors.

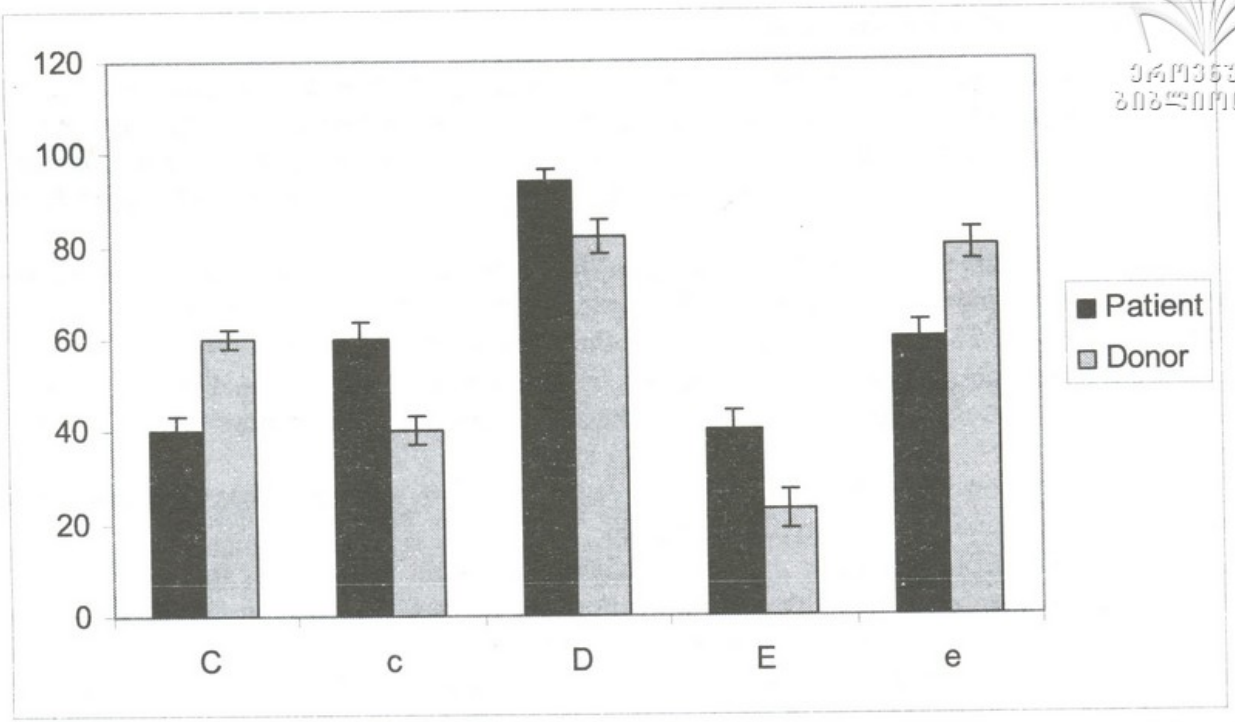

Fig. 2. Occurrence frequency of Rh-system antigens in the patients with breast cancer and donors.

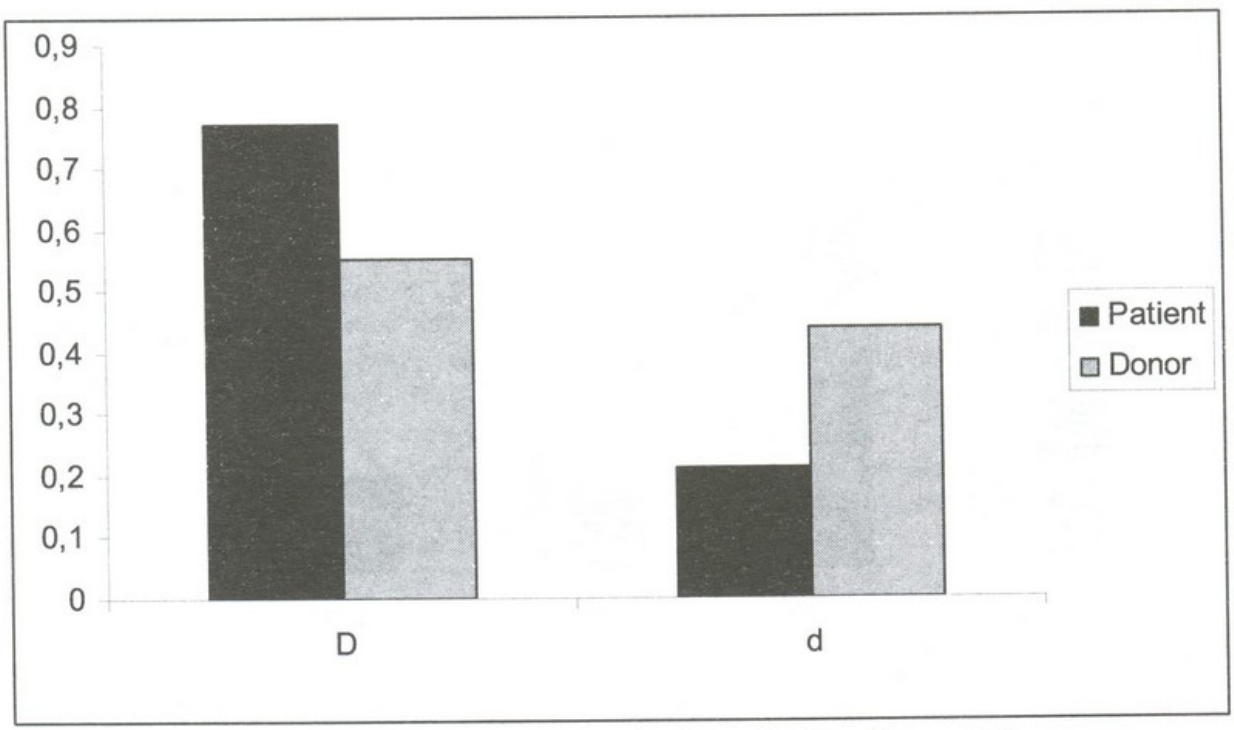

Fig. 3. Concentrations of D- and d-alleles in the diseased and healthy populations.

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ერითროციტული **ABO** და **Rh-Hr** ჯგუფური ანტიგენების კორელაცია სარძევე ჯირკვლის სიმსივნესთან

ნაკაშიძე ი., ნაგერვაძე მ., ხუხუნაიშვილი რ., დიასამიძე ა.

შოთა რუსთაველის ბათუმის სახელმწიფო უნივერსიტეტი

(მიღებულია 15.12.2008)

რეზიუმე

105 სარძევე ჯირკვლის სიმსივნით დაავადებულ პაციენტში და ამავე რაოდენობით ჯანსაღ დონორებში შესწავლილია **ABO, Rh-Hr** ჯგუფური ანტიგენების გავრცელების სიხშირე. ჯგუფური ანტიგენების გამოსავლენად გამოყენებულია იმუნოსეროლოგიური მეთოდები. კვლევებმა აჩვენა სისხლის **A(II)** და **AB(IV)** ჯგუფების მატარებლების მგრძნობელობა აღნიშნული პათოლოგიის მიმართ. სარძევე ჯირკვლის სიმსივნით დაავადებულებში დაფიქსირდა **D** ანტიგენის შედარებით მაღალი გავრცელების სიხშირე (94.24±2.28%) დონორებთან შედარებით (82±3.8%). ასევე დაავადებულ პოპულაციაში გამოვლინდა **E** ანტიგენის მატარებლობის მაღალი სიხშირე.

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AMYLASE-PRODUCING MICROMYCETES ISOLATED FROM SOILS OF SOUTH CAUCASUS

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(Received December 15, 2008)

Abstract

Collection of micellar fungi isolated from different ecological niches of South Caucasus has been created in Durmishidze Institute of Biochemistry and Biotechnology. As a result of screening among collection strains 39 producers of amylase were revealed. Most of them belong to the genus *Aspergillus*. The temperature optimum of three strains was established to be within the range 60°-75°C, making possible to use them in bio and enzymatic technologies to diminish the pollution of the reaction medium while conducting the fermentation process at pasteurization temperature(65°).

Key words: Micromycetes, thermopiles, amylases

Introduction

Amylases are one of the widely spread enzymes in nature. α-amylase (E.C. 3.2.1.1. α-amylase or α-1,4-glucan-glucanohydrolase) hydrolyses α-1,4-type glucan bonds in polysaccharides, which contain more than three glucose residues and are connected with α-1,4- type glucan bonds. α-1,4-amylase reacts with starch, glycogen and other similar poly- and oligosaccharides. This reaction results in synthesis of mix of maltose, glucose and malto-triose [Kvesitadze G., Kvesitadze E., 1999; Amylolytic enzymes, 2006; Kvesitadze et al., 1981].

The mechanism of activity of all known α-amylases is almost the same. They produce unordered hydrolysis of starch, splitting only α-1,4- type glucan bonds in starch molecule, in particular, the bonds between the first carbon atom and oxygen of glucose, joining one glucose atom with another one.

The scheme of α-amylase activity may be expressed like this:

starch

$$\xrightarrow{\alpha\text{-amylase}} \text{dextrans} + \text{maltose} + \text{glucose}$$

glycogen

Among the microscopic fungi active producers of amylases are mostly representatives of the genera Aspergillus and Risopus: A. niger, A. awamori, A. batate, A. phoenicis, A. usamii, A. orizae, A. nidulans, A. foetidium, A. hermebergì (A. niger group), Rh. delemar, Rh. fon Kinensis, Rh. javenicus, Rh. japonicum [Kvesitadze G., Kvesitadze E., 1999; Aquino et al., 2003; Moreira et al., 1999; Pandey et al., 2000].

Microbial amylases become of increased interest for their possible application in some fields of industry, like brewing, bakery, crystal glucose and alcoholic beveriges producing, in medicine – for amylase insufficiency treatment in cases of liver and other deseases.

Producing of stable enzymes is one of the acute problems in bio- and enzyme technology. Conducting of the fermentation processs at pasteurization temperature (65°C) is of great importance as it enables to minimize pollution of the reaction medium. Revealing the strains of amylase producers, which retain activity at high temperature is perspective for solving of this problem.

Materials and Methods

Collection of micellar fungi isolated from different ecological niches of Caucasus has been created in Durmishidze Institute of Biochemistry and Biotechnology. To reveal amylase producer strains among them screening has been performed.

Amylase producers were cultivated at Chapek's modified solid medium (%): $NaNO_3$-9.1, KH_2PO_4-1.0, $MgSO_4 \cdot 7H_2O$-0.5, KCl-0.5, $FeSO_4$-0.02, starch -0,2, agar-2,0 (pH 5.5-6.0).

For submerged cultivation the liquid nutrient medium of the following composition was used (%): starch – 6.0, $NaNO_3$-0.91, KH_2PO_4-0.1, $MgSO_4 \cdot 7H_2O$-0.05, KCl-0.05, $FeSO_4 \cdot H_2O$ - 0.0002, malt sprouts-2.0, pH 5.0-5.5. Submerged cultivation of particular strains of microscopic fungi was performed in 750ml volume conic flasks on thermostat shaker (180-200rot/min) at 30°C for 72h. 10 days old conidial suspension of fungi cultures was used as sowing material. After cultivation the cultural liquid was centrifuged at 4000rot/min and amylase activity was measured in supernatant using the standard method [Amylolytic enzymes, 2006; Rukhliadeva, Goriacheva, 1960].

Results and Discussion

39 producers of amylases were revealed as a result of screening. Among them representatives of the genus *Aspergillus* dominated, but fungi from the genera *Penicillium, Mucor, Rizopus* and *Fusarium* were found also (Fig. 1).

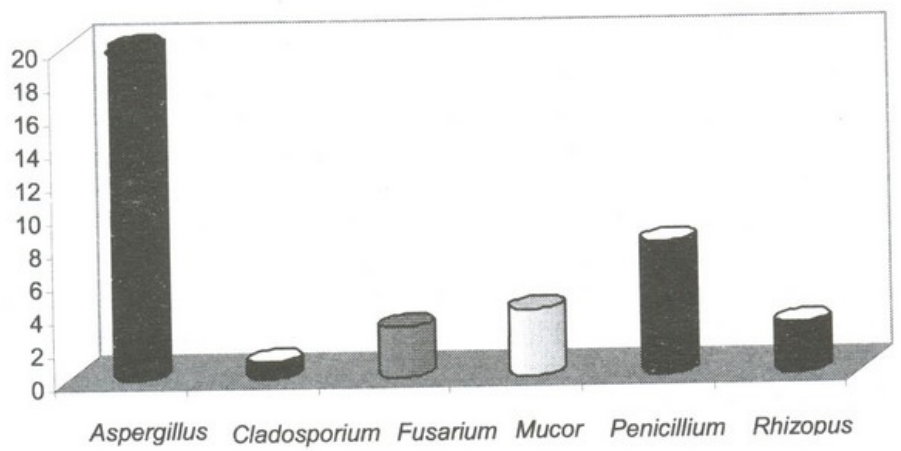

Fig.1. Producers of α-amylase

To determine the extent of extremophilicy of the isolated cultures influence of temperature on their growth and development was investigated. To establish the temperature limits experimental strains were grown on solid nutrient medium at 30°-45°C temperature regime with 5°C intervals. The optimal temperature was determined following the culture increment – according the diameter of the colony and its growth rate. Strains with maximal gain at 20°-35°C were regarded as mesophils, while strains with maximal increase at 40°-42°C - as thermophils. the optimal growth temperature for psychrophils was 5°-10°C, and that of psychrotolerants varied in the range of 5°-20°C, but they grew well at 35°C too [Maheshwari et al., 2000; Kathiresan, Manivannan, 2005] (Fig. 2).

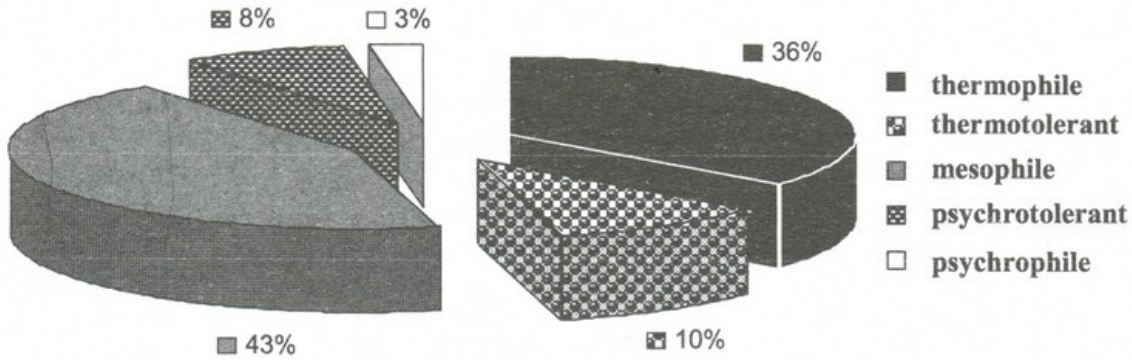

Fig.2 Influence of temperature on growth and development of α-amylase producers

In the cultural liquids obtained after the submerged cultivation of selected strains the amylase activity was studied. For further inveastigations 14 amylase producer strains were selected following two features: hydrolytic activity and thermophilicy. At first amylase producers were tested within 30°-50°C (Table 1).

From the obtained data it is clear that the temperature optimum for most of α-amylases belonging to the isolated cultures varies between 30°-40°C. The optimal temperature of α-amylase belonging to *A. oryzea* 3-9-5 was 30°C. It retained 72.5% of maximal activity at 50°C. The amylase activity of *A. niger* B-5, *A. niger* 6-11, *A. niger* 6-12 increased up to 50°C.

In further experiments the temperature range was raised up to 85°C and amylase activity of those four producers was investigated (Fig. 3).

Obtained results demonstrate that the optimal temperature of activity for α-amylase of *A. niger* 6-11 was 77°C. At 82°C the enzyme revealed 14% of maximal activity, while at 85°C its sharp inactivation took place. In case of *A. niger* 6-12 the temperature optimum of amylase activity was 75°C and at 82°C full inhibition of the enzyme occurred. Optimal temperature of *A. niger* B-5 amylase was 60°C, at 65°C its inactivation began, and at 72°C it fully lost activity. The amylase of *A. orezea* 3-9-5 was fully inhibited at 65°C.

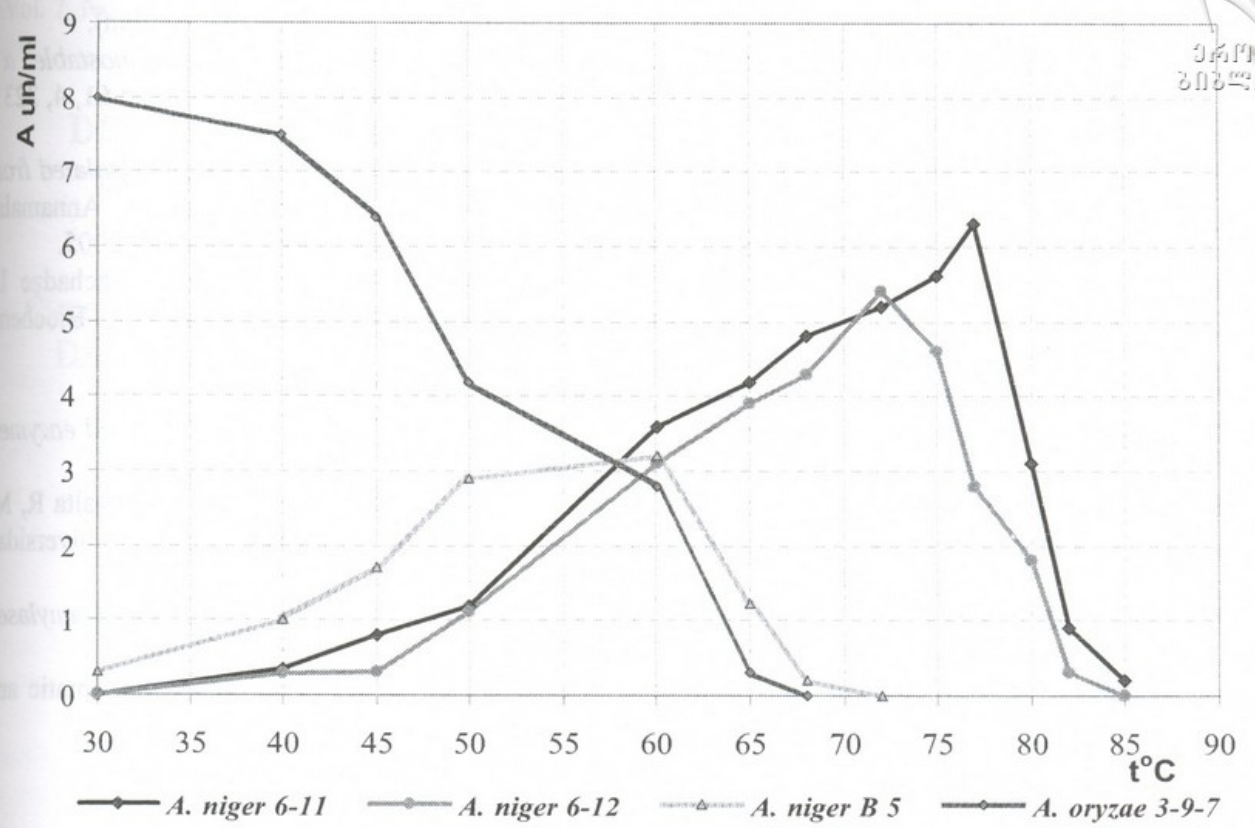

Fig.3. α-amylase activity of *Aspergillus* strains within the limits of 30°-85°C

Table 1. α-amylase producing thermophilic strain

#	α-amylase producers	30°	40°	45°	50°
1	*Aspergillus niger* B-5	0.3	1,0	1,7	2.9
2	*Aspergillus niger* 6-11	-	0.35	0,8	1.2
3	*Aspergillus niger* 6-12	-	0.28	0.3	1.1
4	*Aspergillus niger* B47	2.0	-	-	
5	*Aspergillus oryzae* 3-9-5	8.0	7.5	6.4	4.2
6	*Aspergillus* sp. A1-1	0.9	0.3	-	-
7	*Aspergillus* sp. A2-5	1.8	0.5	-	-
8	*Aspergillus* sp. A6-3	2.0	0.6	-	-
9	*Aspergillus* sp. A7-4	1.5	0.8	0.02	-
10	*Aspergillus* sp. P8-3	1,2	0.5	-	
11	*Aspergillus* sp. P8-5	2.7	2.5	1.0	-
12	*Aspergillus* sp. S 16	3,0	-	-	
13	*Aspergillus* sp. X1-4	1,6	2,5	-	-
14	*Rhizopus* sp. S76	0.8	-	-	

Performed investigation has revealed three producers of α-amylase: *A. niger* B-6 with optimal temperature of enzyme activity at 60°C, *A. niger* 6-11 with optimum at 75°C and *A. niger* 6-12 with optimum at 77°C. According to obtained rersults it is clear that application of α-amylases produced by these micromycetes in different technological processes at pasteurization temperature is quite possible.

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სამხრეთ კავკასიის რეგიონიდან გამოყოფილი ამილაზების პროდუცენტი მოკრომიცეტები

ბურდული თ., ხვედელიძე რ., ურუშაძე თ., ქუთათელაძე ლ. აპლაკოვი ვ.

დურმიშიძის ბიოქიმიის და ბიოტექნოლოგიის ინსტიტუტი

(მიღებულია 15.12.2008)

რეზიუმე

დურმიშიძის ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტში შექმნილია სამხრეთ კავკასიის სხვადასხვა ეკოლოგიური ნიშიდან გამოყოფილი მიცელიალური სოკოების კოლექცია. კოლექციის შტამებს შორის ჩატარებული სკრინინგის შედეგად გამოვლენილია ამილაზების 39 პროდუცენტი, რომელთა უმეტესობა *Aspergillus*-ის გვარს ეკუთვნის. აქედან სამი პროდუცენტის α-ამილაზას მოქმედების ტემპერატურული ოპტიმუმი 60°-75° შეადგენს. ბიოტექნოლოგიასა და ფერმენტულ ტექნოლოგიაში ასეთი ამილაზების გამოყენება პერსპექტიულია ფერმენტაციის პროცესის პასტერიზაციის ტემპერატურაზე (65°C) წარმართვისას სარეაქციო არის დაბინძურების მინიმუმამდე დაყვანის მიზნით.

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DEVELOPMENT OF TECHNOLOGY FOR CREATION OF "OSTEOPHAGE"- A COMPOSITE STIMULATING BONE REGENERATION

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Abstract

Defense of dental arch through filling it with special substances represents a common problem in maxillofacial surgery. It has been determined, that definite species of pathogenic and conditionally-pathogenic microorganisms including *Staphylococcus, E. coli, Streptococcus* and Candida-type fungi in association with these bacteria dominate in alveus. Study of microflora of oral cavity is very important in periodontitis to avoid post-operative complications. Basing on the above-mentioned it is etiologically significant to create an osteorepairing, osteoinductive as well as anti-inflammatory composite, which will contain the substances with all those characteristics. The novelty of the given work is to incorporate bacteriophage into a composite as the best natural non-allergic tool in dealing with the infections, where antibiotics failed. In case of phage application the development of resistance is observed less frequently.

Key words: Bacteriophages, biocomposite, osteophage, Micoliz, mixed infections

Introduction

In recent years a rate of maxillofacial injuries and post traumatic inflammatory complications in alveolar cavity as a result of tooth extraction significantly increased and according to some evidence it reaches 41% pointing to a failure of available preventive remedies. The latter is very important, since antibacterial therapy and creation of the conditions for osteorepairing processes consider to be significant factors in the development of post-traumatic inflammatory complications. Under clinical conditions a risk for transplantant suppuration despite its treatment with antibiotic is high (60.3%).

Tooth extraction is often accompanied by purulent-inflammatory processes, when pathogenic microflora is often isolated from oral cavity. In such cases out of infectious agents gram-positive bacteria (*Staphylococcus, Streptococcus, E.coli, Pseudomonas* etc.) dominate and often are found in association with Candida.

The implementation of wide spectrum antibiotics in medical practice has caused development of resistance in most cases. Schemes for patients' treatment should be divided for those who can be treated within the recognized standards and those who need individual therapy [Kislikh, 1998; Wheat, 2001].

In periodontitis etiologically significant bacteria are mainly isolated in association (60.3%), but among infectious agents Gram-negative bacteria dominate. Their qualitative and quantitative indexes vary. Out of the researching material aerobic bacteria *Staphylococcus epidemidis* (39.34±1.56), *Streptococcus pyogenes* (18.84±1.25%), *Staphylococcus aureus* (10.71±0.99%), *E.coli* (5.66±0.74%) dominate in the microflora. *Candida albicans* in association with microbes is frequently found (34.32%) [Nemsadze, 2006].

In recent years the preparations ("polytome", "Ostim-100", "Bioimplant") produced on the base of Hydroxylapatite and Collagen are widespread [Baier et al., 1986; Ivanov, et al., 1989; Ivanova, Chechina, 2000].

Bacteriophages have been successfully used in dealing with purulent-inflammatory processes in dental care and surgery for many years. Anti-paradental therapeutic remedy "HA-Phage" containing Hydroxyapatite and phage was created in 2002 [Okropiridze, Menabde, 2002]. Combined preparation "Micoliz" was worked out against mixed fungal and microbial infections [Alavidze et al., 2004]. This preparation is characterized by a high activity, it can be used for both, prevention and therapy of mixed infections.

Materials and Methods

Isolation and identification of bacterial strains. Morphological and biochemical identification of all isolates were performed by standard microbiological methods [Pokrovski, 2004].

Isolation and study of Bacteriophages. Phage isolation, titration (Gratia' method), centrifugation, drying and determination of phage activity in mixture was carried out using known methods [Adams, 1961; Gabrilovich, 1968].

Materials for creation of new composite. We used the following materials:

- Brain heart liquid and solid nutrient media; agar of fish, Ploskirev, Sabourand, blood, Endo; antibiotic disks.
- Hydroxyapatite, saccharose, gelatin, propolis, eggshell, methyluracil, nizorale, nystatin, physiologic solution.
- Combined preparation "Micoliz" created on the base of bacteriophages and antifungal substances against mixed fungal and microbial infections at the Eliava Institute of Bacteriophages, Microbiology and Virology, Laboratory of Phage Morphology and Biology.

Bacterial strains. Samples were taken from the area of the teeth gums in dental care units, then inoculated in selective nutrient medium at 37°C for 24-48 h. Biochemical characteristics of bacterial cultures were determined by API-20A (6,0 Merieux-France) system.

Antibiotic (agar diffusion method) and phage sensitivity (lawn dropping method) of a microbe and flora mixed with fungi were studied.

Results and Discussion

Selection of osteoregenerative components of biocomposite

Components of new preparation were selected and the ratios of the composite components were determined. The following types of mixtures were tested:

Hydroxyapatite+Methyluracil+Gelatin; Hydroxyapatite+Propolis+Gelatin;
Eggshell+Propolis+Gelatin; Hydroxyapatite+Propolis+ Collagen.

Selection of phage components of biocomposite

Sensitivity of freshly-isolated bacterial strains to preparation Micoliz was determined. Resistant strains were tested on phage races being available in laboratory and adapted on these strains in case of sensitivity.

New races were included in phage preparation. New phages were isolated from sewage to particularly resistant strains. Then these phages were concentrated and integrated into the preparation "Micoliz".

Technology for Creation of the biocomposite "Osteophage"

At the beginning the effect of various combinations of chemical substances on phages was determined. For this purpose to the different components solved in physiological solution were added the same aliquot of mono, liquid and dry, combined phages. The mixture was 10-fold titrated by Gracia's two-layer agar method up to the dilution when the phage level was at the beginning of the experiment onset (control test).

After obtaining positive results the best combination was selected, various components were added to monophages (*Staphylococcal, Streptococcal* and *E. coli* phages), both to liquid and dry combined bacteriophages. The experiments were carried out consequently by selecting the best composition. Various combinations were checked.

The first combination was: 1. Hydroxyapatite+methiluracil+collagen+bacteriophage

Experimental mixture was added to liquid phage in one case and in another case, to dry phage preparation.

In the following experiments collagen was replaced (as it caused complications connected with testing) by other substance. The following 3 other combinations were tested:

2. Hydroxyapatite+Methiluracil+Gelatin+Bacteriophage
3. Hydroxyapatite+Propolis+ Gelatin+ Bacteriophage
4. Eggshell+Propolis+Gelatin+ Bacteriophage.

Preparation 1. Since collagen didn't need to be swollen, 40-40.4 ml of Micoliz and 13-13.6g of Methiluracil was added to it.

Preparation 2. Gelatin, Hydroxyapatite and Methilutacil were sterilized. Then in laminated receptacle 6-6.8g of Gelatin was smashed and swollen with 40-40.4 ml of Micoliz. The mixture was heated up to 19°C; 6.37g of Methiluracil and 40-40.4g of Hydroxyapatite was added to the obtained mixture.

Preparation 3. Gelatin was swollen with Micoliz using the same aliquot portion as in the second preparation; 13-13.6 g of Propolis and 40-40.4 g of Hydroxyapatite was added to the mixture.

Preparation 4. 6-6.8 g of Gelatin was smashed and swollen with 40-40.4 ml of Micoliz; then 13-13.6 g of Propolis and 40-40.4 g of Eggshell was added to the mixture

Phage activity in all mixtures was defined 1, 24, 48 and 120 hours later by Double-layer agar method and in parallel phage activity was controlled.

The next stage was development of the technology for creating a shape of new complex preparation. For this purpose to the liquid phage with saccharose-gelatin Hydroxyapatite and Methiluracil were added processed by dry sterilization and the mixture was dried (saccharose is necessary for drying).

General toxic, allergic reactions and characteristics stimulating bone regeneration of "Osteophage" inserted into bones of 30 patients were studied [Mamamtavrishvili, 2005].

In the smears taken from 150 patients dominated the following bacteria: *Staphylococcus epidermidis* – 39.34±1.56%; *Streptococcus pyogens* - 18.84±1.25%; *Staphylococcus aureus* - 10.71±0.99%; *Streptococcus mitis* – 9.82±1.52%; *E.coli* pathogenic – 12.42±0.!5%; and in some cases associated flora with *Candida albicans.*

The results of antibioticograms have shown that out of antimicrobial preparation a leading role belongs to Cephalosporin (Table 1). Study of sensitivity of fungous flora has demonstrated that

it is revealed as a monoculture in 6.4% of cases and as mixed culture in 60.3% and that nizorale, lamizin and diflukan represent standard anti-mucosal remedies (Table 2).

Study of phage sensitivity has shown that the strains exhibit the highest percentage of sensitivity toward combined phage preparations proving that phage application is reasonable (Table 3, Fig. 1).

Table 1. Antibiotic Sensitivity

Antibiotics / Strains	Penicillin G	Tetracyclin	Kefzol	Cepazolin	Cyprofloxacin	Rifampicin
Staph.epidemidis	22,5%	49,2%	50,7%	52,5%	78,5%	70,6%
Staph. aureus	25,8%	44,4%	49,5%	46,5%	86,3%	67,7%
Str. pyogenes	31,1%	47,8%	46,5%	51,5%	78,6%	69,1%
Str.mitis	22,2%	45,5%	46%	48,1%	58,9%	65,2%
E.coli	20%	54,5%	47,2%	37%	79%	81,5%

Table 2. Sensitivity of *Candida albicans* against anti-fungal preparations

Diflukan	87,35%
Lamizil	85,37%
Nizoral	80,63%
Nistatin	64,82%

Table 3. Phage Sensitivity

Phages / Strains	Staphylococcus phage	Streptococcus phage	E.coli Phage	Pyophage	Micoliz
Staph.epidemidis	62,9%	-	-	77,6%	79,2%
Staph. aureus	56,7%	-	-	75,9%	78,6%
Str. pyogenes	-	58,4%	-	70,1%	74,2%
Str.mitis	-	53,7%	-	65,8%	69,7%
E.coli	-	-	62,7%	68%	70,9%
Mixed strains	-	-	-	68%	71%

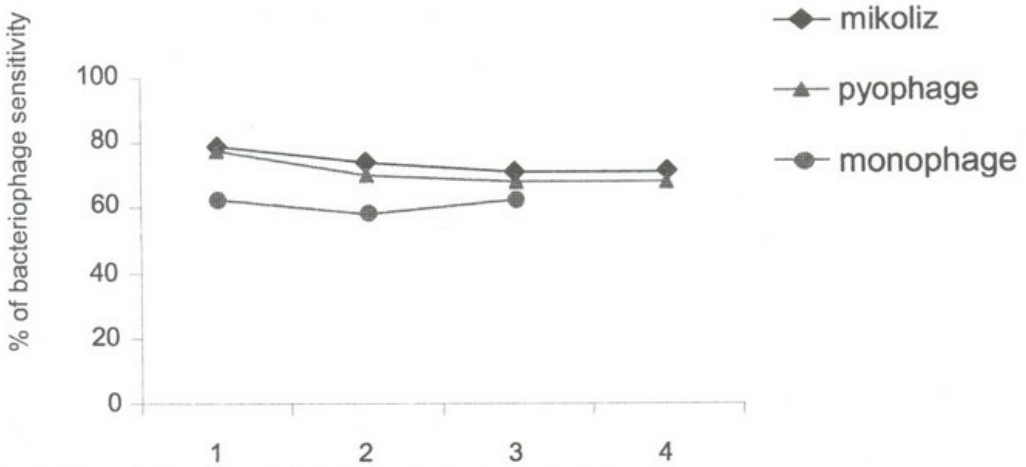

Fig. 1. Sensitivity of bacterial strains to bacteriophages
Bacterial strains (1-Staphylococci; 2-Streptococci; 3- E.coli; 4-mixed strains)

The results of effect of each component on phage are given in Table 4. It turned out that no component inhibited phage and phage maintained its activity for 120 hours.

As a result of studying of four various combinations the best composition of the preparation has been determined: Hydraxyapatite + Methyluracil + Gelatin + Bacteriophage.

The ratios are as follows: 3 : 2 : 0.5 : 2 (in 100ml). Phage maintains its activity in this composition.

Study of dynamics of phage isolation has shown that kinetics of phage release from the composite is the highest. It disintegrated during 54 h (Table 5).

The following composition of a final new composite has been defined:

Hydroxyapatite (40.0) + Methyluracil (13.4) + Gelatin (6.6) + Combined bacteriophage (40.0).

Quantitative ratio of the composite components is the best for achieving optimal outcome and does not suppress phage biological activity.

As a result of composite drying the preparation "Osteophage" has been obtained in a powder form. The studies have proven that preparation is stable during a year under refrigerated at 8-10°C, and thermostated at 37°C conditions ("accelerated getting absolute" method).

Clinical researches have proven that preparation "Osteophage" prevents infection development due to bacteriophage. By means of this preparation dental arch atrophy can be inhibited and stimulation of bone regeneration can be regulated.

Study of microbial flora of tissue around the tooth to be extracted has shown that in this area *Staphylococci, Streptococci*, conditionally-pathogenic *E.coli* and *Candida* type fungi prevail. 62-63% of them are sensitive to the preparation "Osteophage".

Preparation "Osteophage" represents a granular powder which makes its application convenient. Preparation is twice cheaper than its foreign analogous.

Table 4. Phage activity in the Composite

Microbe species	Liquid phage control	Liquid phage + Components (titer in ml)			
		Exposition of Composite (hr)			
Streptococcus pyogenes	$1 \cdot 10^9$	$1 \cdot 10^9$	$1 \cdot 10^9$	$1 \cdot 10^9$	$3 \cdot 10^9$
Streptococcus mitis	$1 \cdot 10^7$	$1 \cdot 10^7$	$1 \cdot 10^6$	$2 \cdot 10^5$	$1 \cdot 10^5$
Staphylococcus aureus	$1 \cdot 10^8$	$1 \cdot 10^8$	$1 \cdot 10^7$	$2 \cdot 10^5$	$1 \cdot 10^6$
Staphylococcus epidermidis	$1 \cdot 10^8$	$1 \cdot 10^8$	$1 \cdot 10^7$	$2 \cdot 10^5$	$1 \cdot 10^6$
pathogenic *E. coli*	$1 \cdot 10^{10}$	$1 \cdot 10^{10}$	$1 \cdot 10^{10}$	$1 \cdot 10^9$	$1 \cdot 10^9$
Microbe species	Dry phage control	Dry phage + Components (titer in ml)			
		Exposition of Composite (hr)			
		1	24	48	120
Streptococcus pyogenes	$1 \cdot 10^7$	$9 \cdot 10^4$	$1 \cdot 10^5$	$1 \cdot 10^5$	$1 \cdot 10^2$
Streptococcus mitis	$1 \cdot 10^6$	$1 \cdot 10^3$	$2 \cdot 10^2$	$1 \cdot 10^2$	-
Staphylococcus aureus	$1 \cdot 10^5$	$1 \cdot 10^4$	$1 \cdot 10^3$	$1 \cdot 10^2$	-
Staphylococcus epidermidis	$1 \cdot 10^5$	$1 \cdot 10^4$	$1 \cdot 10^3$	$1 \cdot 10^2$	-
pathogenic *E. coli*	$1 \cdot 10^{10}$	$2 \cdot 10^5$	$2 \cdot 10^5$	-	-

Table 5. Determination of the critical concentration of phages in the composites. Exposition (hour).

Composite N 2

Microbe species	Liquid phage Control	1	18	36	54
Streptococcus pyogenes	$7 \cdot 10^7$	$19 \cdot 10^5$	$1 \cdot 10^6$	$1 \cdot 10^6$	$13 \cdot 10^6$
Streptococcus mitis	$3 \cdot 10^5$	$2 \cdot 10^5$	$1 \cdot 10^4$	$2 \cdot 10^4$	$1{,}3 \cdot 10^3$
Staphylococcus aureus	$2 \cdot 10^7$	$2 \cdot 10^6$	$2 \cdot 10^5$	$7 \cdot 10^4$	-
Staphylococcus epidermidis	$2 \cdot 10^7$	$2 \cdot 10^7$	$1 \cdot 10^6$	$1 \cdot 10^5$	-
Pathogenic *E.coli*	$5 \cdot 10^7$	$6 \cdot 10^7$	$6 \cdot 10^7$	$5 \cdot 10^7$	$2{,}2 \cdot 10^7$

Composite N 3

Microbe species	Liquid phage Control	1	18	36	54
Streptococcus pyogenes	$7 \cdot 10^7$	$2 \cdot 10^7$	$1 \cdot 10^7$	$2 \cdot 10^7$	$1 \cdot 10^7$
Streptococcus mitis	$3 \cdot 10^5$	$2 \cdot 10^4$	$3 \cdot 10^4$	$1 \cdot 10^4$	$7 \cdot 10^3$
Staphylococcus aureus	$2 \cdot 10^7$	$1 \cdot 10^5$	$1 \cdot 10^5$	$6 \cdot 10^7$	$1 \cdot 10^7$
Staphylococcus epidermidis	$2 \cdot 10^7$	$1 \cdot 10^6$	$1 \cdot 10^6$	$4 \cdot 10^5$	$1 \cdot 10^5$
Pathogenic *E.coli*	$5 \cdot 10^7$	$3 \cdot 10^8$	$7 \cdot 10^8$	$2 \cdot 10^8$	$2{,}2 \cdot 10^8$

Composite N 4

Microbe species	Liquid phage Control	1	18	36	54
Streptococcus pyogenes	$7 \cdot 10^7$	$6 \cdot 10^6$	$3 \cdot 10^6$	$1 \cdot 10^8$	$1 \cdot 10^7$
Streptococcus mitis	$3 \cdot 10^5$	$1 \cdot 10^5$	$2 \cdot 10^4$	$2 \cdot 10^4$	-
Staphylococcus aureus	$2 \cdot 10^7$	$1 \cdot 10^5$	$1 \cdot 10^5$	$5{,}8 \cdot 10^5$	-
Staphylococcus epidermidis	$2 \cdot 10^7$	$1 \cdot 10^6$	$8 \cdot 10^5$	$7 \cdot 10^5$	$1 \cdot 10^1$
Pathogenic *E.coli*	$5 \cdot 10^7$	$2 \cdot 10^7$	$1{,}6 \cdot 10^8$	$2 \cdot 10^7$	$1{,}2 \cdot 10^7$

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ძვლის რეგენერაციის მასტიმულირებელი ახალი ბიოკომპოზიტი "ოსტეოფაგის" შექმნის ტექნოლოგიის დამუშავება

ჭყონია ი.[1], მეიფარიანი ა.[1], ჟღენტი გ.[1], ალავიძე ზ.[1], გოდერძიშვილი მ.[1], ძიძიშვილი მ.[1], ქვათაძე ნ.[1], მამამთავრიშვილი დ.[2], მენაბდე გ.[2]

[1] ელიავას ბაქტერიოფაგიის, მიკრობიოლოგიისა და ვირუსოლოგიის ინსტიტუტი, ფაგების მორფოლოგიისა და ბიოლოგიის ლაბორატორია
[2] თბილისის სახელმწიფო სამედიცინო აკადემია, ყბა-სახის ქირურგიისა და სტომატოლოგიის კათედრა

(მიღებულია 08.09.2008)

რეზიუმე

შექმნილია ანთების საწინააღმდეგო, ოსტეორეპარაციული, ოსტეო-ინტეგრაციული, ოსტეოინდუქციური კომპოზიტი. კომპოზიტის შემადგენლობაში პირველადაა ჩართული ანტიფუნგალურ-ანტიბაქტერიული კომპლექსური ფაგური პრეპარატი, როგორც საუკეთესო ბუნებრივი, არაალერგიული, ინფექციის საწინააღმდეგო საშუალება, რომლის მიმართაც რეზისტენტობის განვითარება ნაკლები სიხშირით მიმდინარეობს. შესწავლილია კომპოზიტის შემადგენელი სხვადასხვა მასალის ურთიერთქმედება ფაგთან. შერჩეულია კომპოზიტის ოპტიმალური ვარიანტი: ჰიდროქსილაპატიტი + მეთილცელულოზა + ჟელატინი + ბაქტერიოფაგი. განსაზღვრულია კომპონენტთა ოპტიმალური თანაფარდობები (2 : 3 : 0.5 : 2). დადგენილია ფაგის გამოყოფისა და აქტივობის შენარჩუნების დინამიკა. იგი 120 საათის შემდეგაც დეინტეგრირებს ნაერთიდან. შემუშავებულია კომპოზიტური მასალის ფორმის შექმნის ტექნოლოგია. შექმნილია პრეპარატი "ოსტეოფაგი", რომელსაც არ გააჩნია უკუჩვენება და ორჯერ იაფია მის უცხოურ ანალოგზე.

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DISTANCE INTERACTION BETWEEN YEAST CELLS *(CANDIDA KEFIR* STRAIN BD2) BY MEANS OF BIOINFORMATION TECHNOLOGY

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Abstract

The fundamental potential of restoration of properties of yeast cells *Candida kefir* strain BD2, lost during the storage has been shown. Usage of bioresonance method allows to restore both, the form and the size of cells, and viability of culture.

Key words: *Candida kefir* strain BD2, bioresonance method, energoinformation signal

Introduction

The chemically mediated communications of microorganisms are actively investigated within the last decade [Kaprelyants, Kell, 1996; Miller, Bassler, 2001]. At the same time it was revealed, that there are also other mechanisms providing interaction and information interchange between microbes. At present it is known, that chemically and spatially divided cultures of microorganisms are capable to influence physiological conditions of each other, leading, in particular, to changes in growth and adhesive properties of cells [Nikolaev, 2000], and modifying light emission of cells [Trushin, 2003].

The specialized equipment based on effect of electromagnetic fields on biological object is already developed and used in practice [Gotovski et al., 2000]. This equipment has played a role of the intermediary in remote interactions between organisms. In other words, physical fields emitted by living organisms can be detected and at the same time, adjusted. The equipments of the center "IMEDIS" (The center of intellectual medical systems) by means of methods of bioresonant and resonance-frequency influences have allowed to receive biological products by information transfer, to raise content of macro-and microelements in eggs without their additional input in forages, to raise productivity and improve properties of cereal cultures without entering into ground the chemical stimulators [Avakova et al., 2008; Kalyuzhny, 2008]. Via energoinformative transfer the growing qualities of lyophilized cultures of lactobacillus have been recovered [Kalyuzhny, 2008]. We have shown the capability of the effect of electromagnetic fluctuations on increase of viability of yeast cells stored in the collection for a long time [Abdulgamidova et al., 2008].

The aim of this work is to study the potential of usage of bioresonance techniques for the restoration of changed or even lost properties of yeast cells *Candida kefir* strain BD2 stored in the collection.

Materials and Methods

As an object of research we used the yeast culture *Candida kefir* strain BD2, stored in the collection of faculty of microbiology of the Baku State University within 2 years in the distilled water at temperature 4-60°C. During the storage the culture was subjected to strong spontaneous changes: viability has sharply decreased and the morphology of cells was changed.

With application of equipments of the center "IMEDIS" the characteristics of yeast cells changed as a result of long storage were recovered. The basis of the method of bioresonance influences (BRI) is the restoration of a natural biorhythm of the system, including all spectra of cellular structures [Bioresonant therapy, 2000]. At the same time the phenomenon of energoinformative transfer of fluctuations of physiological spectra (direct influence) and pathological spectra (inverse influence) with the purpose of restoration of a homeostasis of an investigated organism is considered. Exposition time and the device for realization were chosen optionally.

For experiments carried out in 3 stages, three-day culture of *C.kefir* strain BD2 (initial) and the same culture which has lost some properties because of long storage (damaged) have been selected. The first stage consisted in preparation of suspension of yeast cells of the initial and damaged cultures. As a cultural medium 0.5% physiological solution was used providing the preservation of cell structure without changes.

The second stage consisted in the bioresonance effect on investigated culture in a physiological solution of concentration $72x10^4$ CFU/ml. In this case "direct" effect on initial culture and "inverse" effect on damaged culture was applied. Test tubes with an investigated culture placed in cups for bioresonant influence installed in the equipment "IMEDIS-BRT-program complex". Exposition time - 2 min. Experiment consisted of 3 variants. Experiment 1 represented simultaneously double influence with the spectra on investigated initial (direct influence) and damaged (inverse influence) cultures, which via the equipment was summarized and recorded on the investigated object (direct + inverse influence). Experiment 2 included the direct influence by a spectrum of initial culture, and experiment 3 - inverse influence by a spectrum of the damaged culture.

After influence of electromagnetic fluctuations morphological characteristics was studied [Manual on practical microbiology, 1995] and viability of cells of *C.kefir* strain BD2 was determined [Lusta, Fikhte, 1990]. Results of researches were compared to initial culture and culture after storage not subjected to an irradiation. All experiments were repeated 4 times and statistically processed [Lakin, 1990].

Results and Discussion

With the usage of bioenergoinformation technologies based on bioresonance effect, the potential of recovery of morphological characteristics of yeast cells *C.kefir* strain BD2 stored within 2 years in the distilled water was studied.

Results of research show that the form and the size of *C.kefir* BD2 cells after influences of electromagnetic fluctuations in some experiments were restored up to the condition of initial culture (table 1). The good result was observed only in experiment 1, where the energoinformation signal consists of "direct + inverse" bioresonant influence. At the same time, the oval form of cells was restored in egg-shaped, and the cell sizes have been increased up to initial. In experiment 2, where the energoinformation signal consists only of direct bioresonant influence, the restoration of the cell sizes was observed only. Energoinformation transfer carried out in experiments 3, have not give any appreciable results.

Hence, the restoration of morphological properties of *C.kefir* BD2 occurs effectively by means of bioinformation technologies. The better result is received at simultaneous energoinformation transfer of spectra of the "direct + inverse" influences, than at information transfer separately, which was fulfilled only by 50 % and only in case of information transfer of initial culture.

Table 1. Results of bioresonance effect on restoration of characteristics of *Candida kefir* BD2

№ experiment	Morphological characteristics of culture exposed to irradiation		Index of viability of culture exposed to irradiation
	Cell shape	Cells size (μ)	
1	Egg-shaped	2-4 x 1-2	0.28 ± 0.02
1	Oval	2-3 x 1-2	0.2 ± 0.01
1	Oval	0.01-0,02	0.25 ± 0.01
Initial culture	Egg-shaped	2-4 x 1-2	0.2 ± 0.01
Damaged Culture	Oval	0.05-0,07	0.03 ± 0.002

Effect of electromagnetic fluctuations on restoration of viability of yeast cells *C.kefir* BD2 (table 1) has been studied also. From Table 1 we can see that after the bioresonant influences of electromagnetic fluctuations on yeast cells the results as compared with control are different. In experiments 1 and 3 the viability was not only restored, but even raised 1.4 and 1.25 times in comparison with initial culture. Energoinformation transfer, carried out in experiment 2 has restored the viability of culture up to the condition of initial culture.

Analysis of obtained results shows that the restoration of viability of *C.kefir* BD2 cells by means of bioresonance methods has yielded positive results, and besides a capability of restoration up to the conditions of initial culture the cases of increase of viability were observed.

According to the literary data, transfer of energoinformation signals with the set properties on lactobacillus culture via equipments "IMEDIS" have not only restored the viability, but also have strengthened growing properties of culture after lyophilization [Bioresonant therapy, 2000].

Thus, the fundamental potential of restoration of lost properties of yeast *C.kefir* strain BD2 via boienergoinformation technologies is shown. The optimal variants are picked up. They allow restoring both, the form and the size of cells, and viability of culture.

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დისტანციური ურთიერთქმედება საფუვრის უჯრედებს შორის ინფორმაციული ტექნოლოგიების საშუალებით

განბაროვი ხ., აბიშევი ი., აბდულგამიდოვა ს.

ბაქოს სახელმწიფო უნივერსიტეტი, აზერბაიჯანი

(მიღებულია 10.11.2008)

რეზიუმე

დადგენილია კოლექციაში შენახული საფუვრის *Candida kefir* შტამი BD2-ის დაკარგული თავისებების აღდგენის პრინციპული შესაძლებლობა. ბიორეზონანსული მეთოდების გამოყენება საშუალებას იძლევა აღდგენილ იქნეს უჯრედის როგორც ფორმა და ზომა, ასევე სიცოცხლისუნარიანობა.

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THE STUDY OF MICROORGANISMS PARTICIPATING IN NITROGEN TURNOVER IN THE SOILS OF SHIDA KARTLI (EAST GEORGIA)

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Abstract

A quantitative content of nitrifiers, denitrifiers, nitrate-reducings and free-living nitrogen fixatives has been studied in some soils of Shida Kartli. The physiological groups of dominant microorganisms participating in biogenic turnover of nitrogen in these soils was determined, as well as the main biochemical ways of mobilization and dissimilation of bound nitrogen. It has been established that the soils of Shida Kartli differ from each other by the ways of assimilation and dissimilation of nitrogen which has an influence on the fertility of the soils, as well as on qualitative and quantitative content of cenosis formed on the soils.

Key words: nitrogen fixatives, nitrifiers, denitrifiers, nitrate-reducings, nitrogen turnover

Introduction

The soil appears to be not only a vital environment for different microorganisms but also a product of their vital activity. There are both eukaryotic and prokaryotic organisms in it. A complicated interrelation is established between them, which stability and dynamics prompt the physical-chemical parameters of the soil. The latter together with other abiotic or biotic factors provides a stability and originality of existent biocenosis and agrocenosis.

During the last period a study of qualitative and quantitative content of microflora in the soil attracts a great attention which allows studying the changes in abiotic, biotic and anthropogenic factors under the influence in the soil.

In this respect specific determination of the microorganisms participating in nitrogen mobilization and assimilation is especially important, as well as their quantitative and qualitative ratio in the cenosis including a balance and direction of the processes.

Earlier the microflora of some soils of Georgia was studied [Mamulashvili et al., 2002; Buliskeria et al., 2005; Mamulashvili, Lomtatidze, 2004].

The goal of our investigation was to study a qualitative and quantitative content of the microorganisms participating in nitrogen turnover of some virgin soils of one of the regions of Georgia - Shida Kartli.

Materials and Methods

The content of microflora of the following soil types of Shida Kartli has been investigated: *Cinnamonic Leached, Alluvial Calcareous, Meadow Cinnamonic and Cinnamonic Calcareous*. The samples of soils were taken from the following areas: Ateni, Karaleti, Kheltubani, Nadarbazevi.

The moisture was determined using a weight method in %. The preparation of soil suspension was done according to well known method used in microbiological practice [Tepper, Shilnikova, 2004].

A quantitative determination of the microorganisms of I and II phases of nitrifiers was accomplished on Vinogradski media. The determination of free-living aerobic and anaerobic nitrogen fixative microorganisms was carried on according to Vinogradski method by the isolation on soil lumps and special selective media. The determination of denitrifier and nitrate-reducing microorganisms was done on Giltai and Beriozova nutrient media respectively [Tepper, Shilnikova, 2004].

A quantitative evaluation of the microorganisms was done by the usage of McKred tables [Tepper, Shilnikova, 2004].

Results and Discussion

The content of moisture in the studied soils has been investigated. An amount of moisture in the soil samples is given on the Fig. 1.

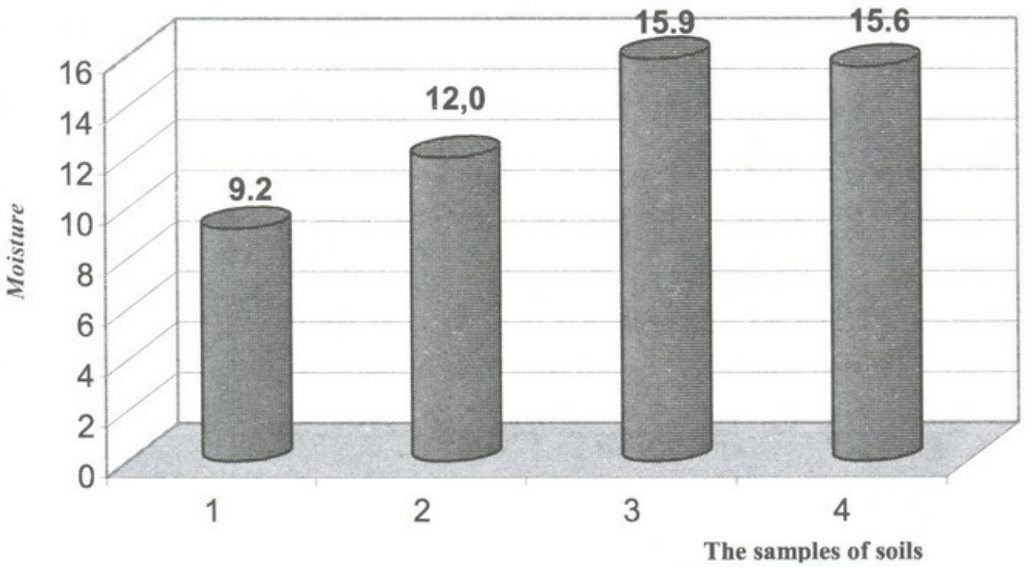

Fig. 1. The amount of moisture in some soil samples of Shida Kartli in %.1 - Cinnamonic Leached; 2 - Alluvial Calcareous; 3 - Meadow Cinnamonic; 4 - Cinnamonic Calcareous

The analysis of the results given on Fig. 1 has shown that by this period (end of September) an amount of moisture is sufficiently high and varies from 9 to 16% in all the four soil types. This index is the lowest in *Cinnamonic Leached* type, which is conditioned by scarcity of plants growing on it. The amount of moisture is comparatively high in *Alluvial Calcareous* soil, is the highest and even equal in *Meadow Cinnamonic* and *Cinnamonic Calcareous* soils.

The nitrate-reducing microorganisms play a very important role in the fertility of the soils. Particularly, they transfer nitrate nitrogen in ammonium ion which is much available for the plants.

A quantitative content of nitrate-reducing microorganisms in some soils of Shida Kartli is given on Fig. 2.

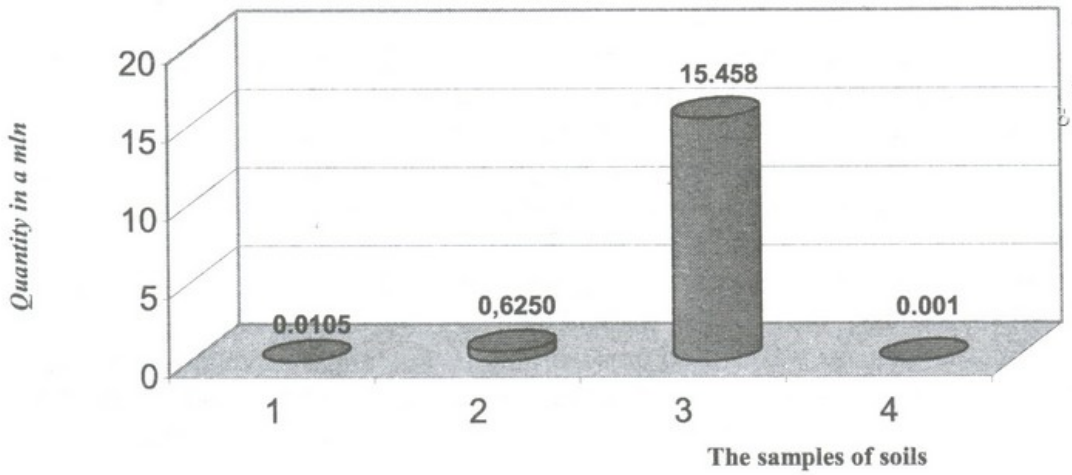

Fig. 2. The amount of nitrate-reducing microorganisms in some soil samples of Shida Kartli in a million. 1 - Cinnamonic Leached; 2 - Alluvial Calcareous; 3 - Meadow Cinnamonic; 4 - Cinnamonic Calcareos

The analysis of the results given on Fig. 2 has shown that these microorganisms are mostly presented in *Meadow Cinnamonic* soils where their amount is approximately 15.5 million per 1 g of dry soil. This index points to the fact that in this soil type mobilization of nitrates for assimilation processes takes place in great amounts, which in its turn indicates high content of nitrate nitrogen as a source of ammonia in *Meadow Cinnamonic* soils.

As compared to *Meadow Cinnamonic* soils, nitrate-reducing microorganisms are 25 times lesser in *Alluvial Calcareous* soils and 1480 times lesser in *Cinnamonic Leached* soils. The microorganisms having nitrate-reducing ability were observed in *Cinnamonic Calcareous* soils in an insignificant amount.

The nitrifiers appear to be one of very significant groups of microorganisms in the soils. At first they transfer ammonia ions to nitrites and then - to nitrates. Despite the fact that nitrogen is more available for the plants in the form of ammonia ion, the mobility of such nitrogen is very low. The ammonia ions are instantly sorbed by the humus, while nitrate nitrogen dissolved in the water freely moves through the soil and are easily found in the system of plant roots.

A general quantitative content of nitrifier microorganisms in some soils of Shida Kartli is given on Fig. 3.

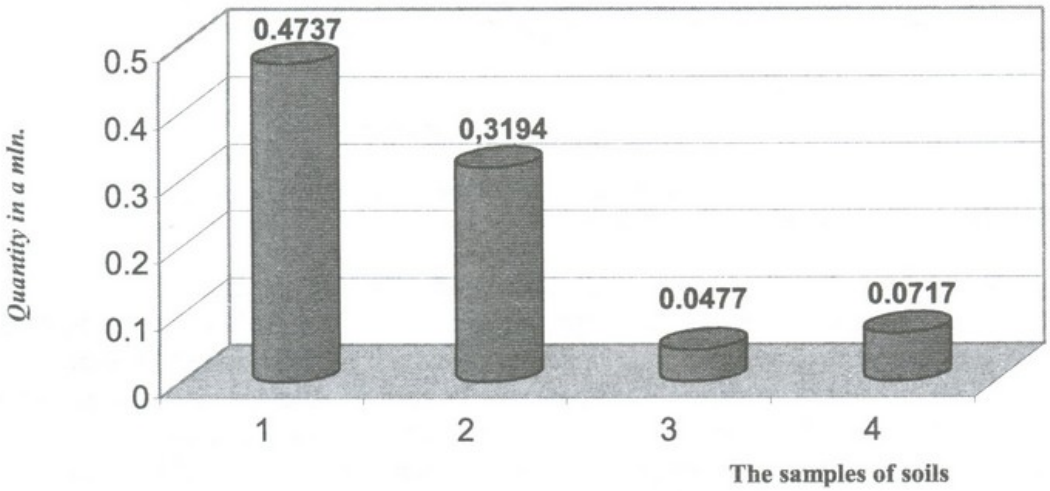

Fig. 3. A general quantitative content of nitrifier microorganisms in some soil samples of Shida Kartli in a million. 1 - Cinnamonic Lleached; 2 - Alluvial Calcareous; 3 - Meadow Cinnamonic; 4 - Cinnamonic Calcareous.

As is seen from Fig. 2 the microorganisms having an ability of nitrification, i.e. an ability of transfer ammonia ions into nitrates and nitrites are mostly presented in *Cinnamonci Leached* and *Alluvial Calcareous* soils, in particular $39x10^6$ and $31x10^6$ in 1 g of dry soil, correspondingly. They are relatively less ($0.06x10^6$) in *Cinnamonic Calcareous* type soils. In a minimal amount nitrifiers are presented in *Meadow Cinnamonic* soils ($0.04x10^6$).

The process of nitrification in the soils takes place in two stages. At the first stage ammonia ion is oxidized up to nitrites, while at the second stage - up to nitrates. Accordingly, the microorganisms having an ability of nitrification are divided into two groups - nitrose and nitrobacteria.

Proceeding from the above said, a quantitative content of nitroses and nitrobacteria has been studied in some soils of Shida Kartli. The results are given on Fig. 4.

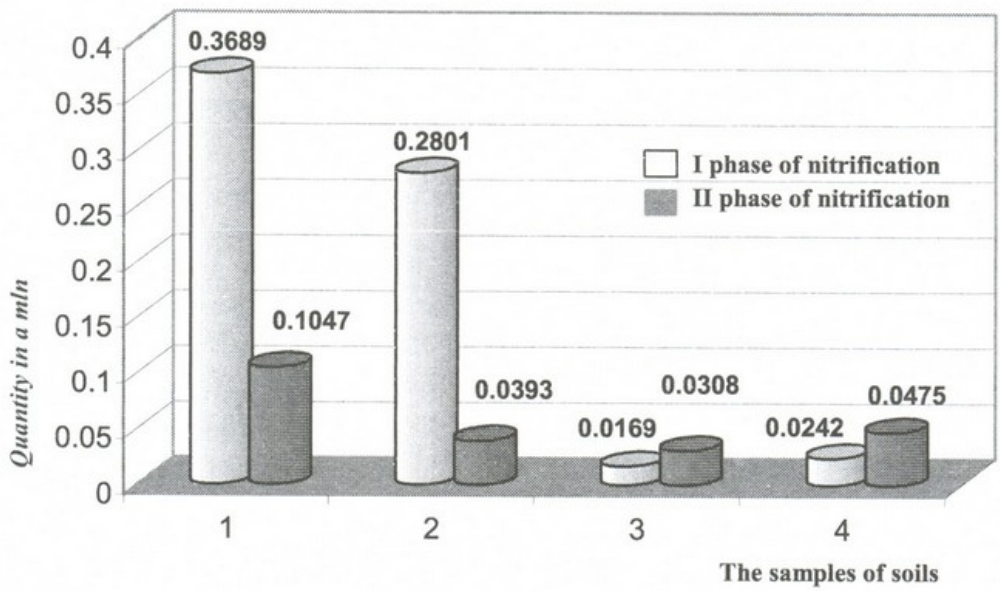

Fig. 4. A general amount of the microorganisms of I and II phases of nitrification in some samples of the soil of Shida Kartli in a million. 1 - Cinnamonic Leached; 2 - Alluvial Calcareous; 3 - Meadow Cinnamonic; 4 - Cinnamonic Calcareous.

The analysis of the results given on Fig. 4 has shown that in *Cinnamonic Leached* and *Alluvial Calcareous* soils the bacteria of I phase of nitrification prevail among the nitrifier microorganisms. In particular, in the first case it prevails 3.5 times, in the second - 7 times, while in *Meadow Cinnamonic* and *Cinnamonic Calcareous* soils the bacteria of II phase of nitrification prevail 2 times as compared to the microorganisms of I phase of nitrification.

The data obtained have shown that in *Cinnamonic Leached* and *Alluvial Calcareous* soils nitrogen of ammonium is mainly mobilized in nitrites, while in *Meadow Cinnamonic* and *Cinnamonic Calcareous* soils - in nitrates.

Denitrifiers are always associated with the undesirable processes for the fertility of the soils. The process of denitrification always begins in the soils at once with the formation of anaerobic conditions (swamping, destruction, etc.) and transfer nitrate nitrogen into gaseous form. This process is known as a loss of nitrogen from the soils.

A quantitative content of denitrifier microorganisms in some soils of Shida Kartli is given on Fig. 5.

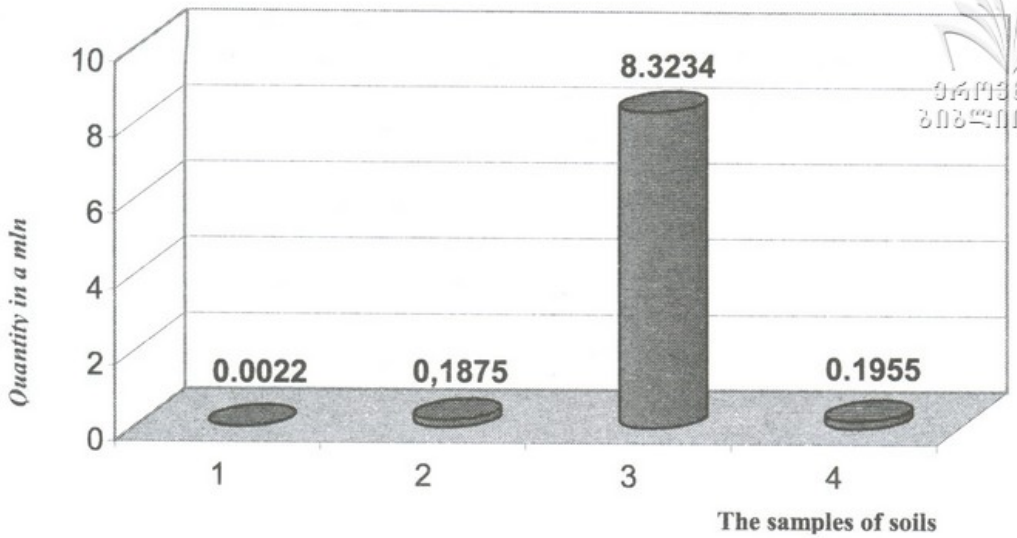

Fig. 5. A general amount of the denitrifier microorganisms in some samples of the soil of Shida Kartli in a million. 1 - Cinnamonic Leached; 2 - Alluvial Calcareous; 3 - Meadow Cinnamonic; 4 - Cinnamonic Calcareous.

As is seen from Fig. 5 content of denitrifier microorganisms is very low in *Cinnamonic Leached* soils and does not exceed 2200 bacteria in 1 g of dry soil, which points to a high level of aeration in these soil types. An amount of denitirifiers is high and even equal in *Alluvial Calcareous* and *Cinnamonic Calcareous* soils. This parameter is very high in *Meadow Cinnamonic* soils and reaches approximately 8.3 mln cells which points to a high loss of nitrogen in these soils.

The microorganisms having an ability of nitrogen fixation play a very important role in the supply of the soils with biogenic nitrogen. Their quantitative index appears to be a paramount parameter of soil fertility. The results are presented on Fig. 6.

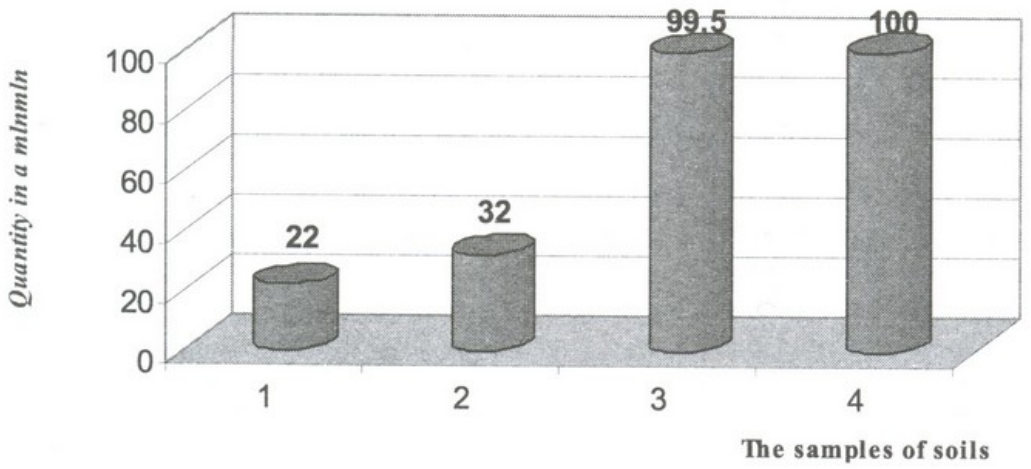

Fig. 6. A general amount of aerobic nitrogen fixative microorganisms in some soil samples of Shida Kartli. 1 - Cinnamonic Leached; 2 - Alluvial Calcareous; 3 - Meadow Cinnamonic; 4 - Cinnamonic Calcareous

The analysis of the results given on Fig. 6 has shown that *Meadow Cinnamonic* and *Cinnamonic Calcareous* soil types are especially rich in free-living nitrogen fixative microorganisms. Their number is almost 3 and 4 times less in *Cinnamonic Leached* and *Alluvial Calcareous* soil types.

An amount of anaerobic nitrogen-fixative microorganisms in some soils of Shida Kartli is given on Fig. 7.

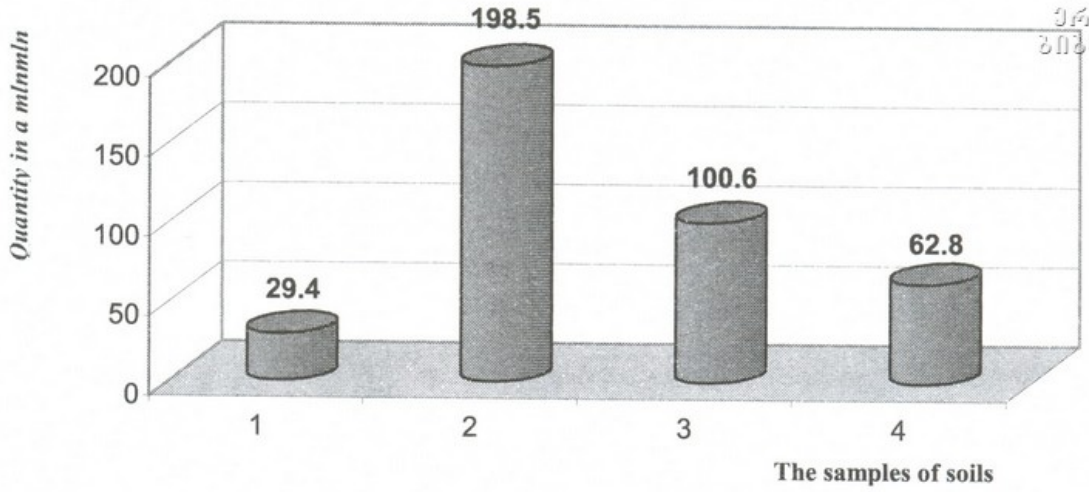

Fig.7. A general amount of anaerobic nitrogen-fixative microorganisms in some soil samples of Shids Kartli. 1 - Cinnamonic Leached; 2 – Alluvial Calcareous; 3 – Meadow Cinnamonic; 4 – Cinnamonic Calcareous.

The analysis of the results given on this figure has shown that an amount of anaerobic nitrogen-fixative microorganisms of Shida Kartli soils is very small and does not exceed few tens of cells in 1 g of dry soil. They are mostly presented in *Alluvial-Calcareuous* soils. An average index was observed in *Meadow Cinnamonic* soil, while in *Cinnamonic Calcareous* and *Cinnamonic Leached* soils their amount decreases even greater and reaches a minimal grade.

Anearobic nitrogen-fixative microorganisms are mainly presented by three species of *Cloctridium* family, as well as by *pasteurianum, butyricum* and acetobutyricum.

A quantitative ratio of these three species in some soils of Shida Kartli is presented on Fig. 8.

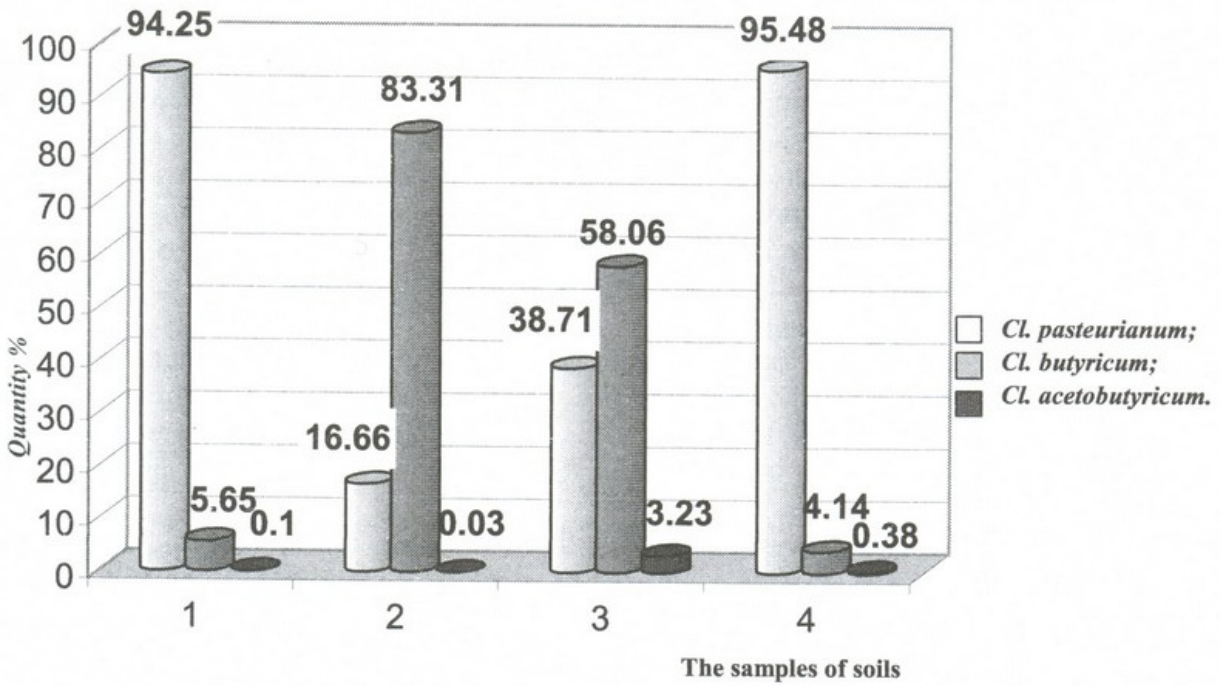

Fig. 8. A quantitative ratio of anaerobic nitrogen-fixative microorganisms in some soil samples of Shida Kartli. 1 - Cinnamonic Leached; 2 - Alluvial Calcareous; 3 - Meadow Cinnamonic; 4 - Cinnamonic Calcareous

As is seen from Fig. 8 percentage of *Cl. acetobutyricum* is insignificant and approximately does not exceed 3%. In *Cinnamonic Leached* and *Cinnamonic Calcareous* soils the anaerobic nitrogen-fixatives are mainly presented by *Cl. pasteurianum*, while in *Alluvial-Calcareous* and *Meadow Cinnamonic* soils - by *Cl. butyricum*. However, a share of *Cl. pasteurianum* is rather high in the latter.

Proceeding from the data obtained it is possible to fulfill a comparative characterization of Shida Kartli soils taking into account a quantitative content of the microorganisms participating in nitrtogen biogenic turnover. Thereto a general amount of the bacteria of each physiological group was taken for 100% in all the investigated soils and a share of these microorganisms for each type soil was calculated (Fig. 9).

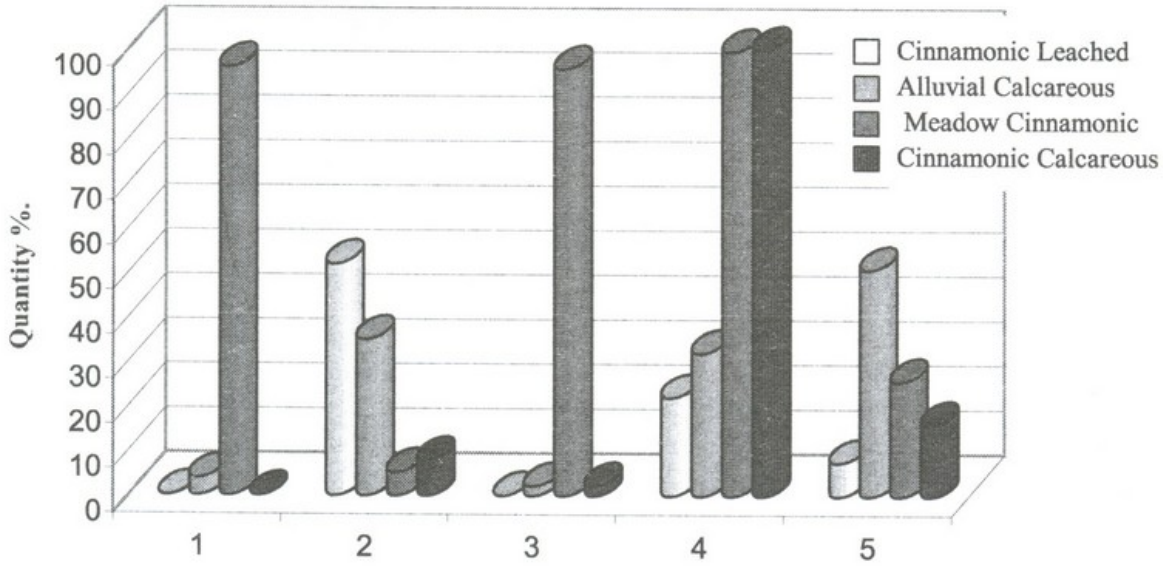

Fig. 9. A quantitative content of the microorganisms participating in nitrogen biogenic turnover in some soils of Shida Kartli. 1. nitrate-reducings 2. nitrifiers; 3. denitrifiers; 4. aerobic nitrogen-fixatives; 5.anaerobic nitrogen-fixatives

The analysis of these results has shown that in *Cinnamonic-Leached* type soil the nitrifiers, aerobic and anaerobic nitrogen-fixatives mainly participate in nitrogen biogenic turnover, while an amount of nitrate-reducings and denitrifiers is insignificant. This points to the fact that in such soil types a mobilization of nitrogen mainly takes place at the expense of atmospheric nitrogen which in future is transformed into nitrite and nitrate nitrogen by means of nitrifiers.

The same processes occur in *Alluvial Calcareous* soil types, although as distinct from *Cinnamonic Leached* soils an insignificant loss of nitrogen takes place on which indicates relatively high content of denitrifiers and nitrate-reducings.

In case of *Meadow Cinnamonic* soils nitrogen is mainly mobilized only at the expense of microbiological fixation of atmospheric nitrogen, to which points very high percentage of aerobic and anaerobic nitrogen-fixatives. At the same time as a result of denitrification a great quantity of nitrogen mobilized in such a way is lost or used for the assimilation metabolism of nitrate-reducing microorganisms.

As compared to other investigated soils, in *Cinnamonic Calcareous* soils atmospheric nitrogen fixed by aerobic and anaerobic nitrogen-fixatives is accumulated in the soil and used insignificantly by nitrate-reducing and denitrifier microorganisms. At the same time a certain part of this nitrogen removes to nitrites as a result of nitrifiers' vital activity.

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შიდა ქართლის ზოგიერთ ნიადაგში აზოტის წრებრუნვაში მონაწილე მიკროორგანიზმების შესწავლა

გოროზია ი., რამიშვილი ნ., კოტია ნ., ბულისკერია ი., ლომთათიძე ზ.

თბილისის ბოტანიკური ბაღი და ბოტანიკის ინსტიტუტი, მიკრობიოლოგიის განყოფილება

(მიღებულია 14.07.2008)

რეზიუმე

შესწავლილია შიდა ქართლის ზოგიერთ ნიადაგში ნიტრიფიკატორების, დენიტრიფიკატორების, ნიტრატმარედუცირებლებისა და თავისუფლადმცხოვრები აზოტფიქსატორების რაოდენობრივი შედგენილობა. დადგენილია ამ ნიადაგებში აზოტის ბიოგენურ წრებრუნვაში მონაწილე დომინანტი მიკროორგანიზმების ფიზიოლოგიური ჯგუფები, ასევე ბმული აზოტის მობილიზაციისა და დისიმილაციის ძირითადი ბიოქიმიური გზები. დადგენილ იქნა, რომ შიდა ქართლის ნიადაგები მნიშვნელოვნად განსხვავდება ერთმანეთისაგან აზოტის ასიმილაციისა და დისიმილაციის გზებით, რაც აისახება ამ ნიადაგების ნაყოფიერებასა და მათზე ფორმირებული ცენოზების თვისობრივ და რაოდენობრივ შემადგენლობაზე.

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NEW DATA ON MICROFUNGI ASSOCIATED WITH WOODY LEGUME PLANTS IN TBILISI ENVIRONS

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Abstract

The paper deals with the new data concerning microfungi on dead branches of legume plants (*Albizia julibrissin, Caesalpinia gilliesii, Cercis siliquastrum, Colutea orientalis, Gleditsia sinensis, Pueraria lobata*) in Tbilisi environs.

Key words: Tbilisi environs, microfungi, legume plants

Introduction

The paper is a continuation of the results of studies on systematic composition of microfungi associated with woody legume trees [Churgulia-Shurgaia, Kacheishvili-Tavartkiladze, 2005]

Materials and Methods

The material investigated includes specimens collected in Tbilisi environs (TE) and Tbilisi Botanical Garden (TBG) in 2005-2008. Identification of collected specimens was conducted on the basis of microscopic analysis of fungal fruit bodies with account of structural morphological features. For identification of fungi classic and modern guide books were used.

Results and Discussion

The report concerns the fungi species which have not yet been recorded in Georgia on legume plants under consideration.

Albizia julibrissin
On dead branches
Coniothyrium fuckelii Sacc. [Grove, 1937].Tbilisi, Saburtalo, University street, 08.06. 2008.

Cytoplea insitiva (Sacc.) Petr. (Syn.: *Coniothyrium insitivum* Sacc.) [Guide Book, 1971] in association with *Alternaria alternata* (Fr.) Keissl. Tbilisi, Didube,24.06.2005.

Cladosporium herbarum (Pers.) Link [Ellis, 1971]. Tbilisi, Saburtalo, University street, 08.06.2008.

Cytospora pruinosa (Fr.) Sacc. [Gvritishvili, 1982]. Tbilisi, Didube, 24.06.2005.

Caesalpinia gilliesii

On dead branches

Macrophoma sophorae Kant. (Kantschaveli,1928). (Conidia 23-30x7-8 μm). TBG, 08.05.2007.

Microdiplodia poincianae Gucev. [Merezhko, 1980]. TBG, 17.02.2005.

Tuberculariа nigricans Link [Sacc.,1886]. TBG, 14.05.2007.

Torula herbarum (Pers.) Link. TBG, 07.09.2006.

Cercis siliquastrum

On dead branches

Camarosporium aequivocum Sacc. [Grove, 1937]. TBG, 19.02.2007.

Camarosporium spartii Trail [Grove, 1937]. Tbilisi, Saburtalo, University street, 28.01.2007.

Diatrypella quercina (Pers.) Nitschke [Traverso, 1906] in association with *Camarosporium aequivocum* Sacc. TBG, 19.02.2007.

Diplodina gleditsiae Hollos. [Guide Book, 1971]. TBG, 18.06.08.

Macrophoma sophorae Kant. (Kantschaveli, 1928). (Conidia 20-30x5-8μm) in association with *Diplodia siliquastri* Westend. TBG, 13.09.2008.

Microdiplodia siliquastri (Pass.) Sacc. [Guide Book, 1971].TBG, 18.06.08.

Microsphaeropsis olivacea (Bonord.) Höhn. [Sutton, 1980]. Tbilisi, Saburtalo, University street, 28.01.2007.

Trimmatostroma betulinum (Corda) Hughes [Ellis, 1971]. Tbilisi, Saburtalo, University street, 28.01.2007.

Colutea orientalis

On dead branches

Allanthozytiella caraganae Danilova [Sutton, 1980]. Tbilisi, Saburtalo, 11.02.2006.

Eutypella grandis (Nitschke) Sacc. [Traverso, 1906]. Tbilisi, Delisi, 24.05.2006.

Diplodia coluteae Schnabl [Merezhko,1980]. Tbilisi, Delisi, 24.05.2006.

Microdidlodia microsporella (Sacc.)Allesch. [Guide Book, 1971] in association with *Cucurbitaria coluteae* (Rabenh.) Auersw. Tbilisi, Delisi, 11.02.2006.

In addition to new records of fungi listed *Phomopsis occidentalis* (Sacc.) Sacc. [Uecker, 1988] on dead stems of *Gleditsia sinensis* and *Phomopsis* sp. (conidia 13-20x3.3-5μm) on *Pueraria lobata* from TBG (04.12.2006 and 22.05.2007, respectively) must be noted.

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ახალი მონაცემები თბილისის შემოგარენის პარკოსან მცენარეებთან ასოცირებული მიკროსოკოების შესახებ

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(მიღებულია 17.12.2008)

რეზიუმე

სტატიაში წარმოდგენილია მოკლე ცნობები იმ მიკროსოკოების შესახებ, რომლებიც აქამდე არ იყო აღნიშნული აბრეშუმა აკაციაზე, გილიესის ცეზალპინიაზე, არღავანზე, ფუჭფუჭაზე, ჩინურ გლედიჩიაზე და პუერარიაზე.

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THE PRELIMINARY RESULTS OF PALYNOLOGICAL INVESTIGATIONS OF LOWER SARMATIAN DEPOSITS OF KAKHETI

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Abstract

The Lower Sarmatian deposits of Kakheti have been studied by palinological method for the first time. About 152 elements of flora are determined. They belong to trees, ferns and grasses. Whole palynological complex is divided into groups in which the plants similar by their ecology are united.

Key words: Lower Sarmatian, Kakheti, palynology.

Introduction

The Lower Sarmatian deposits are widely distributed on the territory of Eastern Georgia. They are mainly connected with Transcaucasus intermontane depression [Gamkrelidze, 2000] and are characterized by frequent changes of facies. On the territory of Kartli Lower Sarmatian is represented by coastal deposits but in Kakheti by sediments of deeper sea.

There are not data about Early Sarmatian flora of Kakheti. The big remains of plants are described only from Middle Sarmatian deposits of this region [Chelidze, 1972, 1987]. In composition of Norio flora 25 forms are determined, from which one is fern, two belong to conifers and the rest to angiosperms. At whole the flora is of subtropical character.

Materials and Methods

By method of palynological analysis 56 samples from Lower Sarmatian deposits from Gombori section were studied. The age of layers is established by data of foraminifers determined by L. Maissuradze (oral report).

Nearly in all samples number of pollen and spores was enough for calculation the percentage of separate taxa in composition of palynocomplexes. These data were put on diagram which reflects on the one hand, the percentage of trees, ferns and grasses and on other hand, the participation in vegetation cover of different groups of plants.

Results and Discussion

In composition of Lower Sarmatian flora of Gombori section 152 forms, belonging to 116 genera and 70 families were determined. The list of plants is given bellow:

Sphagnum sp., *Lycopodium serratum* Thunb., *Lycopodium* sp., *Selaginella* sp., *Ophioglossum* sp., *Osmunda* sp., *Todea* sp., *Schizaeaceae* gen.indet., *Anemia* sp., *Mohria* sp., *Lygodium digitatum* Presl., *L.multivallatum* (Kr.) Ram., *Lygodium* sp., *Cryptogramma* sp., *Pteridacidites verus* (N.Mtchedl.) Sh.et St., *P.grandifoliiformis* St. et Sh.,, *Pteris* sp., *Anogramma* sp., *Pityrorgamma* sp., *Onychium* sp., *Clavifera cf.tuberosa* Bolch., *Clavifera* sp., *Gleichenia* sp., *Gleicheniaceae* gen.indet., *Polypodium* sp., *Polypodiaceae* gen.indet., *Pyrrosia* sp., *Hymenophyllum* sp., *Cibotium* sp., *Dicksonia unitotuberata* Purc., *Dicksonia* sp., *Alsophyla* sp., *Cyathea* sp., *Hemitelia* sp., *Dryopteris* sp., *Woodsia* sp., *Marsilea* sp., *Ginkgo* sp., *Dacrydium* sp., *Podocarpus* sp., *Phyllocladus* sp., *Araucaria* sp., *Abies alba* Mill., *Abies* sp., *Cathaya* sp., *Cedrus* sp., *Keteleeria* sp., *Picea* sp., *Pinus* sp., *Pseudolarix* sp., *Pseudotsuga* sp., *Tsuga canadensis* (L.) Carr., *Tsuga diversifolia* (Maxim.)Mast., *Tsuga* sp., *Sciadopitys* sp., *Cryptomeria* sp., *Cunninghamia* sp., *Sequoia* sp., *Taxodiaceae* gen.indet., *Cupressaceae* gen.indet., *Ephedra* sp., *Comptonia* sp., *Myrica* sp., *Carya* sp., *Cyclocarya* sp., *Engelhardia* sp., *Platycarya* sp., *Pterocarya* sp., *Juglans cinerea* L., *Juglans regia* L., *Juglans sp., Juglandaceae* gen.indet., *Salix sp., Alnus sp., Betula sp., Carpinus betulus* L., *Carpinus* sp., *Corylus* sp., *Castanea* sp., *Castanopsis* sp., *Fagus* sp., *Quercus* sp., *Fagaceae* gen.indet., *Ulmus foliacea* Gilib., *Ulmus* sp., *Zelkova* sp., *Ulmaceae* gen.indet., *Eucommia* sp., *Moraceae* gen.indet., *Caryphyllaceae* gen.indet., *Chenopodiaceae* gen.indet., *Liriodendron* sp., *Magnolia megafigurata* (W.Kr.) Ram., *Magnolia* sp., *Lauraceae* gen.indet., *Annona* sp., *Papaver* sp., *Platanus* sp., *Nuphar* sp., *Nymphaea* sp., *Corylopsis* sp., *Disanthus* sp., *Fortunearia* sp., *Fothergilla* sp., *Hamamelis* sp., *Parrotia* sp., *Sycopsis* sp., *Liquidambar* sp., *Hamamelidaceae* gen.indet., *Rosaceae* gen.indet., *Acacia* sp., *Geranium* sp., *Simarubaceae* gen.indet., *Rhus* sp., *Acer* sp., *Sapindaceae* gen.indet., *Ilex* sp., *Icacinaceae* gen.indet., *Euonymus* sp., *Staphylea* sp., *Vitis* sp., *Tilia* sp., *Sterculiaceae* gen.indet., *Elaeagnus* sp., *Myrtaceae* gen.indet., *Onagraceae* gen.indet., *Alangium* sp., *Nyssa* sp., *Cornaceae* gen.indet., *Dendropanax* sp., *Fatsia* sp., *Hedera* sp., *Araliaceae* gen.indet., *Apiaceae* gen.indet., *Rhododendron* sp., *Ericaceae* gen.indet., *Sapotaceae* gen.indet., *Symplocos* sp., *Fabaceae* gen.indet., *Plantago* sp., *Scabiosa* sp., *Knautia* sp., *Dipsacaceae* gen.indet., *Artemisia* sp., *Asteraceae* gen.indet., *Cercidiphyllum sp., Poaceae* gen.indet., *Nipa sp., Arecaceae* gen.indet., *Tricolporopollenites wackersdorfensis* Thiele-Pfeiffer.

According to the diagram (Fig. 1) the main part of palynocomplex consists of the woody plants and ferns. The participation of grasses is very low. Whole composition of complex we divided into several groups, in which the taxa similar by their requirements towards the climatic conditions were joined.

The first and not numerous is the group of temperate conifers: *Abies, Picea* and *Tsuga.* The last genus is presented by single pollen grains in composition of palynocomplex.

On diagram after temperate conifers the percentage of *Pinus* is given. We separate this genus because pine is intrazonal plant with very wide ecology and probably its distribution was not restricted by definite cenosis.

In the next group the thermophilous conifers are united*: Dacrydium, Podocarpus, Phyllocladus, Cedrus,* representatives of family *Taxodiaceae.* The genus *Ginkgo* is also in this group.

In group of warm-temperate leaf-bearing plants the following genera were joined: *Ulmus, Zelkova, Carpinus, Juglans, Carya, Pterocarya, Corylus, Alnus, Fagus,* some species of *Quercus.*

The most numerous is the group of subtropical leaf-bearing plants, to which are referred *Magnolia, Liriodendron, Nyssa, Alangium,* representatives of families *Arecaceae, Araliaceae,*

Juglandaceae, Fagaceae, Hamamelidaceae, Lauraceae, and others. At whole in composition of flora the participation of warm-temperate and subtropical plants was nearly equal.

The ferns were represented mainly by subtropical forms: *Anemia, Lygodium, Mohria,* representatives of family *Gleicheniaceae.* The number of spores of genera *Pteris* and *Polypodium* in composition of palynocomplex was comparable low.

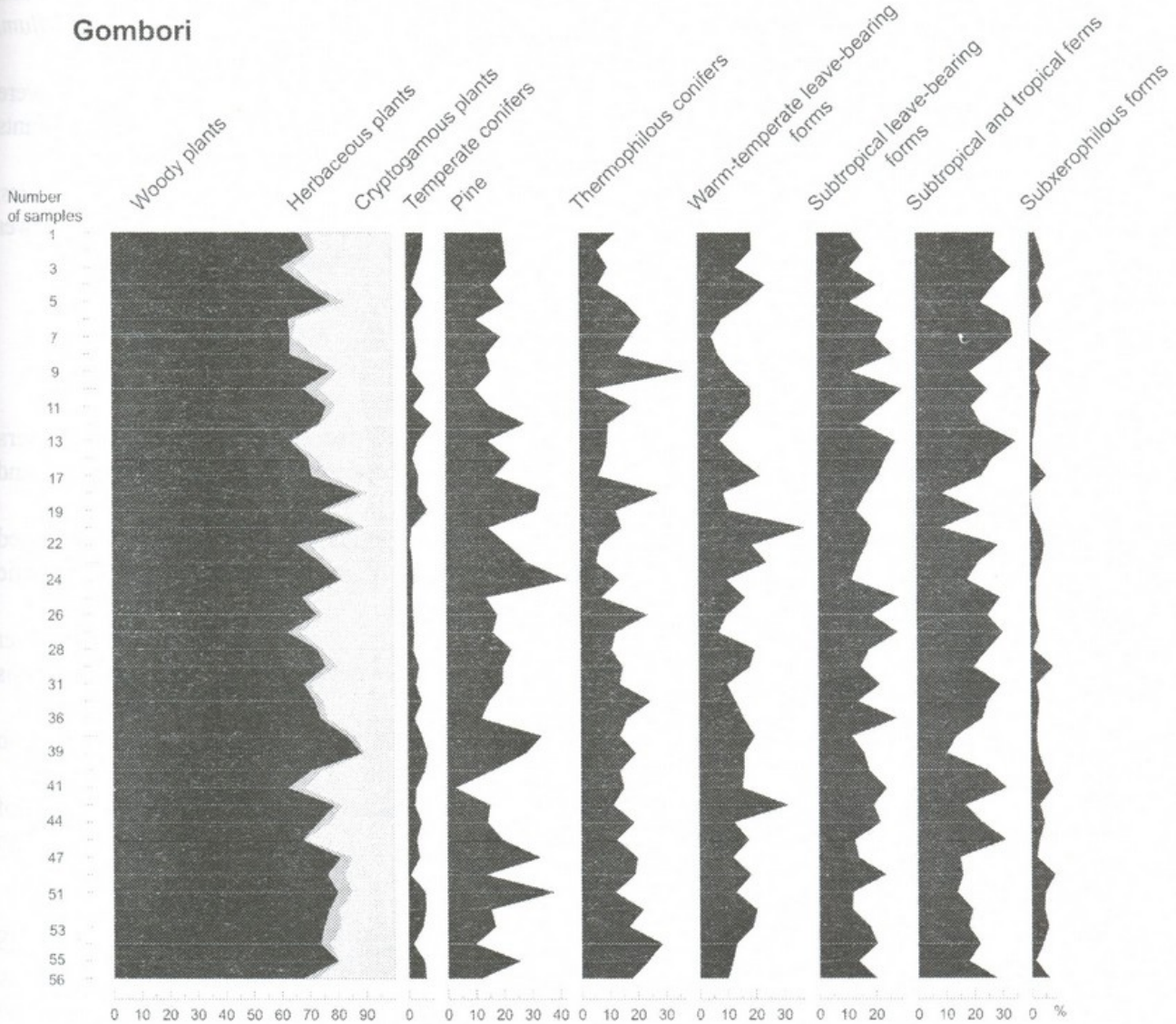

Fig.1. The pollen diagram of Lower Sarmatian deposits of Gombori (Eastern Georgia, Kakheti).

In the last group subxerophilous plants are joined: representatives of genus *Ephedra* and grasses of families *Chenopodiaceae* and *Asteraceae.* At whole in composition of flora the part of this plant group was small.

So, the palynological complex of Lower Sarmatian deposits of Gombori section reflects the existence of polydominant forests. In some localities the cenosis of subtropical trees dominated, in other ones cenosis of warm-temperate plants. The vegetation was distributed on complex relief, which altitude by geological data was not more than 300-500 m [Milanovsky, 1963; Tsagareli, 1980].

The cenosis of thermophilous, moisture-loving plants occupied the sites nearest to the sea, where the representatives of families: *Magnoliaceae, Arecaceae, Araliaceae, Hamamelidaceae,*

Lauraceae, the genera *Myrica, Engelhardia, Platycarya, Castanopsis*, evergreen species of *Quercus*, dominated. The lower layer of this cenosis formed ferns: *Anemia, Lygodium, Gleichenia, Mohria, Cyathea, Dicksonia.* Some of them were the arborescent forms.

On higher levels, where the temperature was lower, plants of the following genera dominated: *Juglans, Zelkova, Carpinus, Quercus, Castanea.* Together with leaf-bearing plants thermophilous conifers were growing: *Dacrydium, Podocarpus, Cedrus*, some of *Taxodiaceae* and genus *Ginkgo.* The representatives of families: *Poaceae, Apiaceae* and ferns *Pteris, Polypodium, Anogramma, Pyrrosia*, composed the grass layer of these cenosis.

Along rivers riparian and swamp forests were distributed, which main components were *Liquidambar, Nyssa, Ulmus, Alnus, Carya*, some of *Hamamelidaceae.* The cryptogamous plants were represented mainly by bog moss.

The territories situated far from see were covered by cenosis of dark-conifer plants: *Abies, Picea* and *Tsuga.* They occupied very small area that can be explained by absence in Lower Sarmatian conditions favorable for wide development of plants of temperate climate.

Conclusion

In composition of Lower Sarmatian flora of Kakheti 152 forms belonging to 116 genera and 70 families are determined. The main part of paleoflora is represented by the subtropical and warm-temperate plants: trees and ferns.

The polydominant forest was the main formation of vegetational cover. It was composed from separate cenosis which distribution was depended on the character of relief, microclimatic conditions and edaphic factors.

The warmest and the most humid sites were occupied by subtropical cenosis. At higher levels, where the temperature was lower, warm-temperate leaf-bearing plants and thermophilous conifers were distributed.

The temperate dark-conifer cenosis occupied small area situated far from accumulation basin.

The development of riparian and swamp cenosis was dependent mainly on the character of soil. Very likely that they were distributed on different levels of relief and their composition changed in accordance with microclimatic conditions.

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კახეთის ქვედა სარმატული ნალექების პალინოლოგიური კვლევის წინასწარი მონაცემები

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(მიღებულია 08.12.2008)

რეზიუმე

კახეთის ქვედასარმატულ ფლორაში განსაზღვრულია 152 ფორმა, რომელიც მიეკუთვნება 116 გვარს და 70 ოჯახს. ფლორის მთავარ ნაწილს სუბტროპიკული და სითბოზომიერი მცენარეები – ხეები და გვიმრები შეადგენს. მცენარეული საფარის ძირითადი ფორმაცია იყო ცალკეულ ცენოზებად დაყოფილი პოლიდომინანტური ტყე, მცენარეული ცენოზების გავრცელება განპირობებული იყო რელიეფის ხასიათით, მიკროკლიმატური პირობებით და ედაფური ფაქტორებით. ყველაზე თბილი და ნოტიო ადგილები სუბტროპიკულ ცენოზებს ეკავა. ჰიფსომეტრულად ზემოთ, სადაც ტემპერატურა უფრო დაბალი იყო, იზრდებოდა სითბოზომიერი ფოთლოვანი მცენარეები და თერმოფილური წიწვოვნები. ნალექდაგროვების აუზიდან მოშორებით, მცირე ზომის არეალი ეკავა ზომიერი ჰავის მუქ-წიწვოვან მცენარეებს. სანაპირო ზოლის და ჭაობის ცენოზების გავრცელება, ძირითადად, განპირობებული იყო ნიადაგის ხასიათით. სავარაუდოდ, ისინი რელიეფის სხვადასხვა დონეებზე იზრდებოდნენ და მათი შემადგენლობა მიკროკლიმატური პირობების შესაბამისად იცვლებოდა.

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BIOEVENTS ON THE TERRITORY OF GEORGIA DURING THE LATE CENOZOIC AS EVIDENCED BY FORAMINIFERS AND PALYNOMORPHS.

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Abstract

The foraminifers and palynomorphs from the Late Cenozoic in Eastern and Western Georgia were studied in more than 45 sections. The evolution of the foraminifers is traced and development stages are distinguished for the Sarmatian and the Meotian. The landscape-phytocenological method allows interpreting the evolution of vegetation depending on climatic fluctuations. Common turning-points in the development of marine and terrestrial biocoenoses are recognized and connected with changes in paleogeography and depositional environments in the region of today's Caucasus during the Late Cenozoic.

Key words: Georgia, Late Cenozoic, foraminifers, palynomorphs.

Introduction

The Late Cenozoic deposits of Georgia accumulated in Transcaucasus intermontane depression, which in Early and Middle Sarmatian times was entirely covered by the sea. In the Late Sarmatian, which was characterized by important orogenic movements, this depression was transformed into dry land with two bays – the Kurian Bay, which was connected with the Caspian Sea, and the Rionian Bay, which was the part of Black Sea. Beginning from the Late Sarmatian and during most of the Pliocene, the territory of Georgia belonging to the Kurian Bay was dry land. Only in the Late Pliocene (Akchagilian, Apsheronian) the marine regime was restored here.

To the west, in the Rionian Bay, marine conditions prevailed during the entire Late Miocene, Pliocene and Pleistocene and the accumulation of Black Sea deposits was continuous. Today it is the stratotypical region of the Eastern Paratethys where the Late Cenozoic deposits are fully represented and well characterized by marine fauna.

The transformation of the region adjacent to the Black Sea into isolated high mountain ranges occurred in the Late Sarmatian. Throughout the Late Cenozoic, this so-called Colchis refuge was the area where elements of the Tertiary flora survived.

Material and Methods

The objectives of this investigation were the identification of assemblages of foraminifers and palynomorphs from Late Cenozoic deposits of Georgia. Sections of Sarmatian marine sediments occur in the whole of Georgia, but marine deposits of the following stages are found mainly in Western Georgia.

The following Sarmatian sections were studied:

Eastern Georgia: Nadarbazevi, Uplistsikhe, Gombori, Phrone River, Aragvi River, Iori River, Satschenisi Ravine, Baramaant-chevi Ravine, bore-holes Eldari 1, Taribana 39 and 40, Vashliani 1 and 10, Karasi 1.

Western Georgia: Galidzga River, Djgali, Vake, Khobi, Bakhioti, Opcha, Olori, Tebene, Khobis-tskali River, Ochkhamuri, Kvirila River, Joboura and others.

The marine deposits of younger stages were studied in the following sections in Western Georgia: Japhareuli, Atapi River, Gedjiri River, Gudou River (Meotian); Mokvi River, Gogoreti, Kulistskali River, Urta, Zana (Pontian); Duabi River, Gogoreti, Orapho (Kimmerian); Khvarbeti, Tsikhisperdi, Nagobilevi (Kujalnician); Khvarbeti, Archeuli, Tsiagubani, Shava (Gurian); Chakhvata River, Nakhveta River, Tsvermagala, Djumati (Chaudian); Ureki (Old Euxinian); Tskaltsminda (Uzunlarian); Karangatian, New Euxinian were studied in material from bore-holes of the Colchis plain (Fig.1).

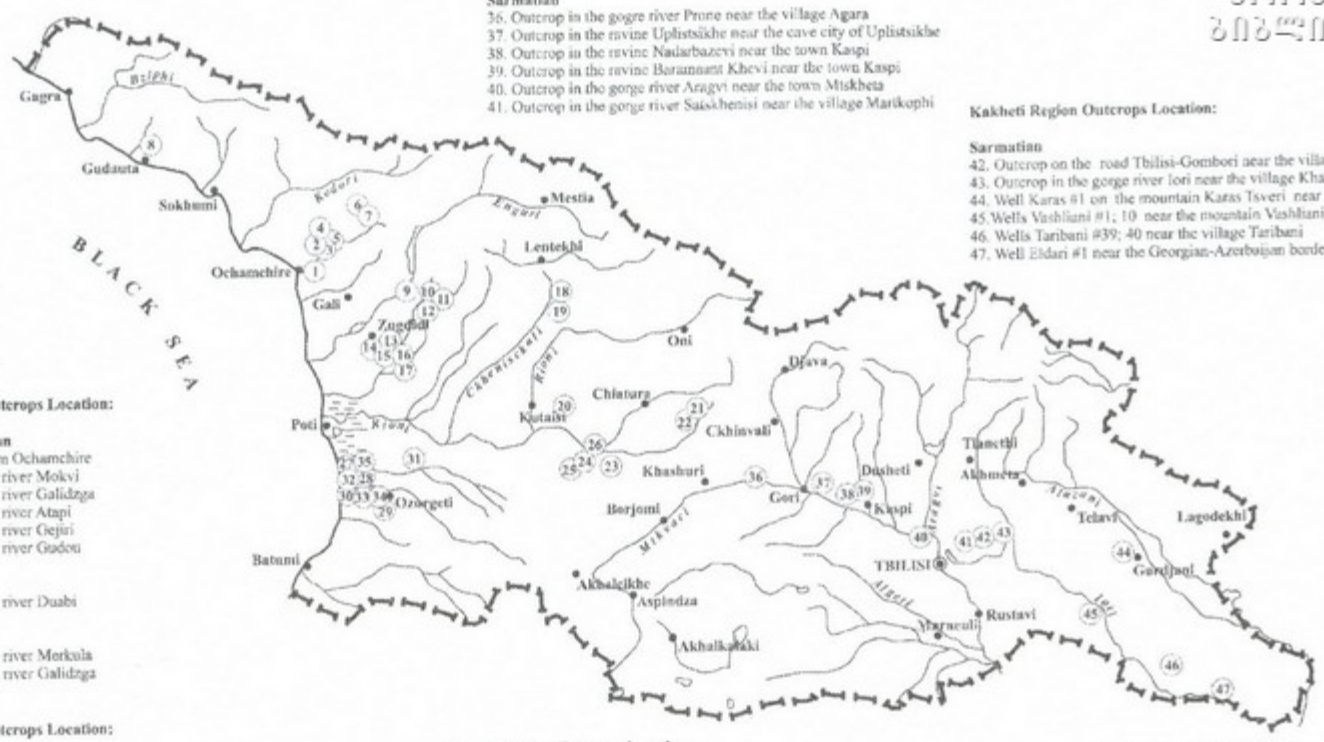
The foraminifers both from Eastern and Western Georgia were studied. One to two hundreds grams of the rock was boiled in water until disaggregation. Disaggregated residues were then decanted in water and washed several times under running water. They were then dried in an oven and studied for microfossil content. The residues were placed in a picking tray with a 0.4X0.4 cm grid, identified and placed in micropaleontological slides.

The palynological material was interpreted by the landscape-phytocenological or zonal method [Borzenkova, 1992]. This allows reconstructing forest types according to their vertical distribution as vegetation belts: dark-conifer forest; beech forest, which was formed only in the Early Pleistocene; polydominant forest, composed of subtropical leaf-bearing trees and conifers and also warm-temperate plants. The pine as intra-zonal tree and indicator of humidity was distinguished separately.

Results and Discussion

The Sarmatian deposits are widely distributed on the territory of Georgia. They are characterized by frequent changes of facies, which was the main factor that influenced the composition of foraminifer fauna. During the Late Cenozoic, several stages in the development of foraminifers can be distinguished. The characteristics of these stages are given below.

The Early Sarmatian commonly overlies conformably the Konkian deposits and is lithologically represented by argillo-arenaceous facies (sands, clays). In the shallow water deposits of the lower part of the Early Sarmatian are dominant: *Elphidium macellum* (F.et M.), *E. crispum* (Linne), *E.obtusum* (d'Orb.), *E.hauerinum* (d'Orb.), *E.angulatum* (Egger), *Elphidiella artifex* (Serova). In lesser numbers of specimens occur*:* *Ammonia* ex.gr. *beccarii* (Linne), *Nonion tumidulus* Pish., *N.bogdanowiczi* Volosh., *Sinuloculina consobrina* (d'Orb.), *Variadentella reussi* (Bogd.), *V.sartaganica* (Krash.), *Affinetrina guriana* (O.Djan.), *Porosononion martkobi* (Bogd.), *P. subgranosum* (Egger) and others.



Western Georgia:

Abkhazian Region Outcrops Location:

Sarmatian and Meotian
1. Outcrop near the town Ochamchire
2. Outcrop in the gorge river Mokvi
5. Outcrop in the gorge river Galidzga
6. Outcrop in the gorge river Atapi
7. Outcrop in the gorge river Gejiri
8. Outcrop in the gorge river Gudoti

Kimmerian
4. Outcrop in the gorge river Duabi

Kujalnician
3. Outcrop in the gorge river Merkula
5. Outcrop in the gorge river Galidzga

Samegrelo Region Outcrops Location:

Sarmatian and Meotian
9. Outcrop in the gorge river Oluri near the village Fakhulani
10. Outcrop in the gorge river Chanistskali near the village Djgadi
11. Outcrop in the gorge river Khobistskali near the village Zumi
12. Outcrop in the gorge river Ochkhamuri near the village Lesichine
13. Outcrop in the gorge river Chanisekali near the village Djikhashkari

Meotian and Pontian
14. Outcrop near the village Urta
15. Outcrop in the gorge river Kulisckali near the village Akhalsopeli
16. Outcrop near the village Zeni
17. Outcrop in the gorge river Khobi near the village Zeni

Lechkumi Region Outcrops Location:

Sarmatian and Meotian
18. Outcrope near the village Orbeli
19. Outcrope in the gorge stream Gvirisha near the village Nasperi

Kartli Region Outcrops Location:

Sarmatian
36. Outcrop in the gogre river Prone near the village Agara
37. Outcrop in the ravine Uplistsikhe near the cave city of Uplistsikhe
38. Outcrop in the ravine Nadarbazevi near the town Kaspi
39. Outcrop in the ravine Baramauri Khevi near the town Kaspi
40. Outcrop in the gorge river Aragvi near the town Mtskheta
41. Outcrop in the gorge river Saisikhenisi near the village Martkophi

Eastern Georgia:

Kakheti Region Outcrops Location:

Sarmatian
42. Outcrop on the road Tbilisi-Gombori near the village Ujarma
43. Outcrop in the gorge river Iori near the village Khashmi
44. Well Karas 61 on the mountain Karas Tsveri near the village Cheremi
45. Wells Vashliani #1; 10 near the mountain Vashliani
46. Wells Taribani #39; 40 near the village Taribani
47. Well Eldari #1 near the Georgian-Azerbaijan border zone

Imereti Region Outcrops Location:

Sarmatian and Meotian
20. Outcrope near the village Simoneti
21. Outcrope near the village Bakhioti
22. Outcrope near the village Korbouli
23. Outcrope near the village Vardzia
24. Outcrope near the village Sviri
25. Outcrope in the gorge stream Lukhuta near the village Obcha and Well Obcha #4
26. Outcrope in the gorge river Djoboura near the city Zestaphoni

Guria Region Outcrops Location:

Sarmatian
33. Outcrop near the village Vake

Meotian and Pontian
29. Outcrop near the village Gogoreti
34. Outcrops near the village Tkhinvali
31. Outcrop near the village Jumati

Kimmerian and Kujalnician
29. Outcrop near the village Gogoreti
34. Outcrops near the village Cikhisperdi and in the gorge river Orapho
28. Outcrop near the village Khvarbeti
35. Outcrop near the village Morokhvili

Gurian
28. Outcrop near the village Khvarbeti

Chaudian
34. Outcrops in the gorge river Nakhveta
32. Outcrop near the mountain Tsvermagala

Old Euxinian
30. Outcrop near the village Ureki

Uzunlarian
27. Outcrop near the village Tskaltsminda

Fig. 1. The locations of outcrops of Late Cenozoic deposits of Georgia

In deeper argillaceous deposits of the same age are found: *Varidentella reussi* (Bogd.), *V. sartaganica* (Krash.), *Sinuloculina consobrina* (d'Orb.), *Affinetrina guriana* (O.Djan.), *Cycloforina karreri* (Reuss), *Quinqueloculina collaris* (Gerke at Iss.), *Spiroloculina okrajantzi* Bogd., *Articulina problema* Bogd., *A.tamanica* Bogd. Commonly it is possible to see also thin-shelled, comparatively small *Nonion tumidulus* Pish., *N.bogdanowiczi* Volosh., *Porosononion martkobi* (Bogd.), *P.subgranosum* (Egger), *Fissurina* ex.gr. *marginata* (Walk et Bojts.) and statoliths of crustaceans *Sarmysis sarmaticus* (Khal.). Both facies are characterized by layers with ***Variadentella reussi*** and are attributed to **stage I.**

The upper part of the Early Sarmatian is also characterized by different assemblage the composition of which is dependent on lithology or depth of the basin where the sediments accumulated. In near-shore deposits the species of *Nonion, Elphidium,* and *Porosononion* dominate. In deeper-water deposits miliolids dominate: *Articulina, Varidentella, Sinuloculina, Cycloforina, Affinetrina* and also, rarely, *Nonion* and *Porosononion.* In complexes of this age the first endemic species and subspecies are found: *Sinuloculina consobrina sarmatica* (Gerke), *Cycloforina karreri ovata* (Serova), *C.complanata* (G.at Iss.), *Articularia articulinoides* (G.at Iss.), *Fissurina cubanica* Bogd., *F. elongata* Pobed.

The number of species and individuals in these complexes is higher than in the previous one. The sizes of tests are bigger too. The peculiar sign of many species from deposits of the second part of the Early Sarmatian is increased intra-specific variability. Typical of these deposits are the layers with ***Elphidium aculeatum*** and they correspond to **stage II** in the development of foraminifers.

The Early Sarmatian is overlain by the Middle Sarmatian, which is represented by clays, sandstones with bases of limestones, marls and heterolithic conglomerates. They are dated by specific complexes of foraminifers and divided into three parts: the layers with *Affinetrina voloshinovae*, the layers with *Porosononion aragviensis* and the layers with *Porosononion hyalinum.*

The lower part of the Middle Sarmatian is represented by comparatively deep-water argillaceous deposits. The complex of foraminifers is composed of the following species: *Affinetrina guriana* (O.Djan.), *A.voloshinovae* (Bogd.), *A.voloshinovae caudata* (Bogd.), *A.voloshinovae timenda* (Bogd.), *Sinuloculina angustioris* Bogd., *S. corrugis* (Kolesn.et Gerke), *S. consobrina sarmatica* (Gerke), *Varidentella floriformis* (Bogd.), *V. reussi costulata* n.subsp., *V. nanae* Maiss., *V.nanae megrelica* Maiss., *Cycloforina complanata* (G.at Iss.), *C.delicatulla* (Vella), *C.karreri ovata* (Serova), *Flintina tutkowskii* Bogd., *Articulina problema* Bogd., *A.apscheronica* Bogd., *A.calickii* Bogd., *A.bidentata costata* Bogd., *Articulina articulinoides* (G.at Iss.), *Sarmatiella moldawiensis* Bogd., *S.prima* Bogd., *Dogielina sarmatica* Bogd.et Volosh., *D.kaptarenko* Didk., *Meandroloculina gracilis* Bogd., *M.bogatschovi* Bogd., *M. moldawiensis* Bogd., *Fissurina cubanica* Bogd., *F.elongata* (Pobed)., *F.horrida* (Bogd.), *F.irma* Bogd., *Discorbis obtusum* (d'Orb.), *Nonion tumidulus* Pish., *N. bogdanowiczi* Volosh., *Elphidium macellum* (F.et M.), *E.hauerinum* (d'Orb.), *E.crispum* (Linne), *E.rugosum* (d'Orb.), *Porosononion subgranosum subgranosum* (Egger), *P.subgranosum umboelata* Bogd., *P.granosum*(d'Orb.), *Bolivina sarmatica* Didk., *B.dilatata brevis* Cicha et Zapl., *Bulimina* aff.*elongata* d'Orb. In many cases also the remains of statoliths of *Misidae* can be seen.

In Eastern Georgia, in the same interval in sections on the Phrone River and in the Satskhenisi Gorge, the species of *Porosononion* are more numerous and have big sculptured tests. Large ostracods and otoliths of fishes are dominant. This complex corresponds to the layers with ***Affinetrina voloshinovae*** and to **stage III** in the development of foraminifers.

The next period of the Middle Sarmatian is a time of flourishing of foraminifers. In the middle part of Middle Sarmatian, the sizes of foraminifers increase significantly and the number of Middle Sarmatian endemic forms achieves the maximum. However, in synchronous deposits of

Western and Eastern Georgia the percentages of miliolids and elphidiids are different. In the West, the miliolids are dominant but in the East *Nonionidae* and *Elphidiidae* prevail. The complex of foraminifers in the Middle Sarmatian is composed of the following species: *Affinetrina voloshinovae voloshinovae* (Bogd.), *A.voloshinovae eldarica* n.subsp., *A.voloshinovae pecteniformis* (Bogd.), *Cycloforina complanata* (G.at Iss.), *C.karreri* (Reuss), *Sinuloculina angustioris* (Bogd.), *Varidentella reussi costulata* n.subsp., *Spiroloculina okrajantzi* Bogd., *S. kolesnikovi* Bogd., *Flintina tutkowskii* Bogd., *F.schweyeri* Bogd., *Articulina apscheronica* Bogd., *A. paradoxalis* Bogd., *A. calickii* Bogd., *Dogielina sarmatica* Bogd.et Volosh., *Sarmatiella prima* Bogd., *S.costata* Bogd., *S.moldawiensis* Bogd., *S.subtilis* Bogd., *Meandroloculina litoralis* Bogd., *M.conicocameralis* Bogd., *M.gracilis* Bogd., *M.aculeata* Bogd., *Nonion bogdanowiczi* Volosh., *Porosononion subgranosum subgranosum* (Egger), *P.subgranosum umboelata* Bogd., *P.granosum* (d'Orb.), *P.aragviensis* (O.Djan.), *P.hyalinum* (Bogd.), *Elphidium fichtelianum* (d'Orb.), *E.macellum tumidocamerale* Bogd., *E.joukovi* Serova, *E.rugosum* (d'Orb.), *E. regina* (d'Orb.), *E.regina caucsica* Bogd., *Fissurina horrida*(Bogd.), *F.elongata* (Pobed.), *Bolivina sarmatica* Didk., *Bulimina* sp., *Discorbis* sp. Statoliths of *Mysidae* and *Ostracodae* are also present. This complex is characterized by layers with ***Porosononion aragviensis*** and defines **stage IV** of foraminifer development.

To the end of the Middle Sarmatian, the complex of foraminifers is sharply impoverished: all Middle Sarmatian endemics and species still present from the Early Sarmatian disappear. This impoverished complex is composed of the following species: *Porosononion subgranosum subgranosum* (Egger), *P.subgranosum umboelata* Bogd., *P.aragviensis* (O.Djan.), *P.hyalinum* (Bogd.), *Elphidium fichtelianum* (d'Orb.), *E.macellum* (F.et M.), *E.crispum* (Linne), rare thick-shelled *Affinetrina guriana* (O.Djan.) and also *Ostracodae.* This complex corresponds to layers with ***Porosononion hyalinum*** and to **stage V** of development of Sarmatian foraminifers.

The orogenic movements, which began at the end of Middle Sarmatian, achieved the maximum in the Late Sarmatian and in most of Georgia a continental regime was established. Marine conditions prevailed only in Western Georgia (Guria, Abkhazia, Samegrelo) and in some regions of Kakheti (Eastern Georgia). In the Late Sarmatian deposits, foraminifers are practically absent.

The Late Sarmatian in most of Georgia is represented by continental deposits. In Kartli it is so-called Natskhorian suite, and in the south of Kakheti the Eldarian suite. The marginal- marine deposits of Late Sarmatian age in Eastern (Kakheti) and in Western Georgia are characterized by mollusks: *Mactra caspia* Eichw., *M.bulgarica* Toula, *M.timida* Zhizh. (BuleishviLi, 1964; Gruzinskaya et a1,, 1974) and fresh-water ostracods: *Iliocypris, Cyprideis, Candona* (Gruzinskaya et a1., 1986). Single *Ammonia* is very seldom. Together with ostracods, numerous remains of *Characea* are commonly present.

The Late Sarmatian regression was followed by a transgression in the Meotian. The connection between the Black Sea and the Mediterranean regions was restored, which promoted the penetration of marine fauna into the Eastern Paratethys.

At the beginning of the Meotian, relatively stenohaline species of foraminifers and many other organisms (mollusks, ostracods, bryozoans, corals, algae and others) colonized the basin. The new stages of development of Miocene fauna began. Most of the Meotian species of foraminifers have some similarity with recent species of the Mediterranean Sea and also with Middle Miocene – Sartaganian foraminifers of the Ponto-Caspian region. The Early Meotian complex is composed of the following species: *Quinqueloculina seminulum maeotica* Gerke, *Q. iberiae* Bogd., *Q.akneriana maeotica* Mass., *Q.similiakneriana* Didk., *Cycloforina gracilis* (Karrer), *C.vermicularis* (Karrer), *C.disparilis galidzgensis* (Bogd.), *C.lachesis* (Karrer), *C.aff. postbadenensis* Vengl., *Sinuloculina* ex.gr. *consobrina* (d'Orb.), *S.consobrina maeotica* Maiss., *S.*aff.*brauni* (Reuss), *S.pseudocuneata* (Gerke), *Affinetrina seminulum ukrainica* (Didk.), *A.* ex. gr. *guriana* (O.Djan.), *A. bogatschovi*

(Bogd.), *Triloculina* aff. *intermedia* (Karrer), *T.* aff. *inflata* (d'Orb.), *Spiroloculina* sp., *Miliolinella circularis maeotica* Maiss., *M.*aff.*majuscula* Popch., *Pyrgo clypeata* d'Orb., *Hauerina iljnae* Bogd., *H. tchelidzei* Popch., *H.*ex.gr.*confusa* Serova, *Spirolina elegans maeotica* Didk., *S.*aff.*stelligera* Didk., *Articulina tenella maeotica* Bogd., *Cibicides* sp., *Sigmoilina* sp., *Discorbis* sp., *Ammonia beccarii liliae* Popch., *Nonion* aff. *matagordanus* Kornfeld., *Porosononion* aff. *martkobi* (Bogd.), *P.*aff.*subgranosum* (Egger), *Haynesina maeotica* Maiss., *Elphidium* exgr.*ponticum* Dolgop.et Pauli, *E. macellum maeotica* Gerke, *E.feodorovi* Bogd., *E.mirandum maeotica* Maiss., *Bolivina* aff.*nisporenica* Didk., *B.*ex.gr. *tumida* Cushman, *B.* ex. gr. *variabilis* (Williamson), *B.* ex. gr. *moldavica* Didk., *B. atapica* Maiss., *B. iae* Maiss., *Cyclogira* sp.

Later, in the second part of the Meotian, when the connection with the marine basin was interrupted, began the impoverishment of the complex of Mediterranean species and the distribution of a complex rich in euryhaline species. The Late Meotian complex is composed of: *Elphidium macellum maeotica* Gerke, *E.feodorovi* Bogd., *E.*ex.gr.*ponticum* Dolgop.et Pauli, *Porosononion* aff. *martkobi* (Bogd.), *P.*aff. *subgranosum* (Egger), *Nonion* aff.*matagordanus* Kornfeld., *Ammonia beccarii liliae* Popch., *A.*ex.gr.*beccarii* (L.), *Quinqueloculina seminulum maeotica* Gerke, *Miliolinella* ex.gr.*circularis* (Born.), *Discorbis* sp., *Bolivina* aff.*nisporenica* Didk., *Fissurina* sp.

Consequently, during the Meotian, two stages can be distinguished in the development of foraminifers in Western Georgia, corresponding to the Bagerovian and Akmanaian substages. In the Early Meotian complex, stenohaline species of foraminifers (**stage VI**) dominate, and in Late Meotian euryhaline (**stage VII**) species abound.

The Meotian is overlain by deposits of Pontian age. In some sections of Western Georgia (Atapi River, Bia village), the transition between Late Meotian and Early Pontian is gradual. The lower part of the Early Pontian is represented by the Eupatorian horizon, which is characterized by few species of euryhaline mollusks, ostracods and foraminifers [Taktakishvili, 1975; Arevadze, 1977; Vekua, 1979; Suladze, 1984; Maissuradze, 1980, 1985].

In the Eupatorian deposits, rare specimens of the following forms are present: *Quinqueloculina seminulum maeotica* Gerke, *Elphidium* ex.gr.*ponticum* Dolgop.et Pauli, *Porosononion* ex.gr.*subgranosum* (Egger), *Nonion* aff.*matagordanus* Kornfeld., *Ammonia beccarii* (L.). The ostracods are represented by the following species: *Caspiola venusta (Zal.), Pontoniella acuminata (Zal.), Eucypris ziberi (Mch.).* Among the mollusks *Prosodacna littoralis* (Eichw.) is found. The history of development of Late Cenozoic foraminifers ends with the Early Pontian-Eupatorian (**stage VIII**).

The Pontian deposits, which cover the Eupatorian horizon, accumulated in a closed basin with low salinity. In these deposits, foraminifers are absent. The process of isolation continued until the end of the Pliocene and, therefore, foraminifers are absent in post-Pontian (Kimmerian, Kujalnician, Gurian, Chaudian) basins of the Eastern Paratethys. The biotopes of these basins were inhabited by brackish ostracods, mollusks and other euryhaline organisms. Endemic species of mollusks and ostracods that immigrated from adjoining regions are dominant [Nevesskaya et al., 1986].

In Eastern Georgia the continental analogue of the marine deposits are the Dushetian and Shirakian suites, which are dated as Meotian-Pontian. These deposits are represented by thick conglomerates, with rare interbeds of clays and coarse-grained sandstones.

In the Akhaltsikhian depression, Meotian deposits are represented by volcanic facies (the so-called Goderzian suite). In adjacant regions this is known as the Kisatibian suite. According to their lithological composition, these suites are attributed to the Sarmatian – Meotian (Skhirtladze, 1958).

In the Pliocene of Eastern Georgia, foraminifers are known only from the Middle Akchagilian deposits (Djikia, 1976), the accumulation of which was connected with transgression

and normalization of basin conditions. In the section of Kvabebi, which is known as a locality rich in fossil mammals (Vekua, 1972), the following complex of foraminifers was determined: *Bolivina* aff.*textilarioides* Reuss, *B.aksaisca* Chutz., *B.*aff.*advena* Cushman, *Bolivina* sp., *Buliminella elegantissima* d'Orb., *Cassidulina prima* Suzin, *Discorbis* sp., *Ammonia beccarii* (Linne) (**stage IX**).

On the territory of Azerbaijan (Kurian depression), the fauna of foraminifers is seen in Early Akchagilian deposits (Kadyrova, 1960). This complex is composed of: *Cassidulina crassa* d'Orb., *C.prima* Suzin, *Cibicides lobatulus* (Walk.et Jak.), *Miliolinella* cf.*aksaica* Chutz., *Bolivina kovalevskyi* Agal., *B.*ex.gr.*tarchanensis* Subb.et Chutz., *B.*ex.gr.*advena* Cushm., *Nonion punctatus* d'Orb., *Porosononion subgranosum* (Egger), *Discorbis* aff. *orbicularis* (Terq.), *D. arculus* Chutz., *D. multicameratus* Chutz., *Ammonia beccarii* (Linne). In the upper part of the same section, other microfaunistical horizons with many species of *Bolivina* were identified by Kadyrova (1960). The determination of these forms is not in her work, but the author interprets them as marker for Akchagilian deposits.

In the Caspian region of the Eastern Paratethys, the Akchagilian stage is overlain by Apsheronian deposits, in which foraminifers are absent. For dating the layers ostracods and mollusks are used. The fauna is determined as a brackish to fresh-water complex. The history of development of fauna implies a gradual decrease in salinity, analogue to the basins of Western Georgia (Kujalnician, Gurian, Chaudian).

The complete absence of foraminifers in post-Pontian deposits can be explained at first by a sharp decrease of salinity. In isolated basins of the Pliocene and Pleistocene, according to mollusks, the salinity of the water was in the range of 0.5-5‰. Such conditions of course provoked the extinction of foraminifers, which as euryhaline organisms cannot survive in salinities lower than 5‰ [Iljina et al., 1976].

Except salinity also the strong competition with ostracods must be taken into account, organisms of higher organization and more enduring of low-salinity conditions than foraminifers. Both occupy one and the same biotope and use the same food (phytoplankton and bacteria). The numerous populations of ostracods were widely distributed in the basins of the Pliocene and Pleistocene.

The other factor promoting the extinction of foraminifers was unstable climatic conditions in post-Pliocene epochs. At first it is necessary to note the worsening of climate between the Miocene and Pliocene – in Eupatorian times. Consequently, it can be assumed that decrease in salinity, absence of a stable and warm environment, and shortage of food combined to create unfavorable conditions for foraminifers.

In summary, the history of Late Cenozoic foraminifers in Georgia began in the Sarmatian, when the Eastern Paratethys originated as a wide basin, which had no connection with the open sea. In this isolated and slowly freshening basin all stenohaline species of foraminifers gradually became extinct, which earlier inhabited in Konkian fully saline basin. The ancestors of the Sarmatian foraminifers were Late Konkian (Veselankian) mostly euryhaline species, which supported the progressive freshening and led to the original Sarmatian fauna. The fauna of Sarmatian basins is rich in new endemic genera, species, and ecological subspecies, which underwent the influence of biotic and abiotic factors varying in time and space. The characteristic sign of Sarmatian foraminifers is the small number of families (7) and genera (20), represented by great number of endemic species, subspecies and individuals. The common number of species and subspecies is 120.

The analysis of whole paleontological material shows that the history of Sarmatian foraminifers on the territory of Georgia can be divided into three intervals of time: an early interval, which reflects the process of gradual formation of Sarmatian microfauna; a middle interval with the maximum development of fauna, late interval, when nearly all groups of foraminifers died out due

to unfavorable conditions. These intervals of development of fauna correspond to the three stratigraphical substages of the Sarmatian: Volkhinian, Bessarabian, and Khersonian. In turn they can be subdivided into smaller units by the foraminiferal assemblages.

This, the Volkhynian substage is divided into two parts: lower - the layers with *Varidentella reussi* (**stage I**), and upper - layers with *Elphidiun aculeatum* (**stage II**). The Bessarabian substage is divided into three parts: the layers with *Affinetrina voloshinovae* (**stage III**); the layers with *Porosononion aragvinsis* (**stage IV**) and layers with *Porosononion hyalinum* (**stage V**).

The characteristic sign of the Early Meotian is the development of stenohaline Mediterranean species (**stage VI**), which gradually disappear. The Late Meotian complex is then composed of more euryhaline species (**stage VII**). The extinction of marine stenohaline species was provoked by cessation of contact with a fully saline basin at the end of the Meotian.

In some sections (Atapi, Bia) the transition from Meotian to Pontian (Eupatorian horizon) is gradual. The latter is characterized by small numbers of euryhaline species of mollusks, ostracods and foraminifrs (**stage VIII**). The **stage IX** was established in Middle Akchagilian deposits of Eastern Georgia.

These established stages (Fig.2) can be used for biostratigraphic correlation [Maissuradze, 1966, 1971, 1980; Maissuradze et al., 2004, 2007, 2008; Maissuradze, Koiava, 2006, 2008; Shatilova et al., 2008; Koiava, 2006; Koiava et al., 2008a].

The analysis of palynological material allows to distinguished 12 stages (palynozones) in the history of development of climate and vegetation (Fig. 3). We don't give the detailed description of stages, because their characteristic features can be seen on the diagrams, which are constructed on the basis of palynological investigations of the Late Cenozoic [Shatilova et al., 2004, 2005, 2006, 2007, 2007a, 2008, 2008a; Kvavadze, Rukhadze, 1989, 1999]. We discuss only the turning-points, which had significant influence on the composition of flora and on the dynamics of vegetation.

The first turning-point can be related to the boundary between Miocene and Pliocene (boundary between **stages I** and **II**), when the rich subtropical forest of the Sarmatian and Meotian changed to pine forest at the beginning of the Pontian (Eupatorian horizon), which can be explained by a decrease of humidity (Fig.4). Connected with this time was the mass extinction of many thermophile plants and changes in dynamics of vegetation in Western Georgia. Nevertheless, the main part of the Pontian and Kimmerian flora remains subtropical and warm-temperate, indicating the high enough temperatures and humidity in this region.

The dynamics of vegetation on the territory of Eastern Georgia was quite different. The palynological assemblages in Middle Sarmatian deposits of this region reflect the domination of unstable climatic conditions, with a development towards xerophytisation. The area of pine was wider than in Western Georgia and often prevailed over the area of polydominant forest (Fig. 5). In the second part of the Middle Sarmatian in Eastern Georgia, a reduction of forest and an expansion of grasses began. This process was more pronounced in the region of Kartli where palynology and mammals [Mchedlishvili, Mchedlishvili, 1953; Meladze, 1967] indicate that, after the Middle Sarmatian, the dominant vegetation types were steppe, semi-deserts and deserts.

In Kakheti, the xerophytisation was not so strong. According to the macro-flora [Chelidze, 1972], mainly an increase of Mediterranean elements in the composition of flora took place. The role of these elements became more important in the Early-Middle Pliocene when, according to the Shirakian macro-flora [Kolakovsky, Ratiani, 1967], a hot and dry climate cenosis like maquis and shibliak (deciduous bush formations in the Mediterranean region) prevailed on the territory of Kakheti.

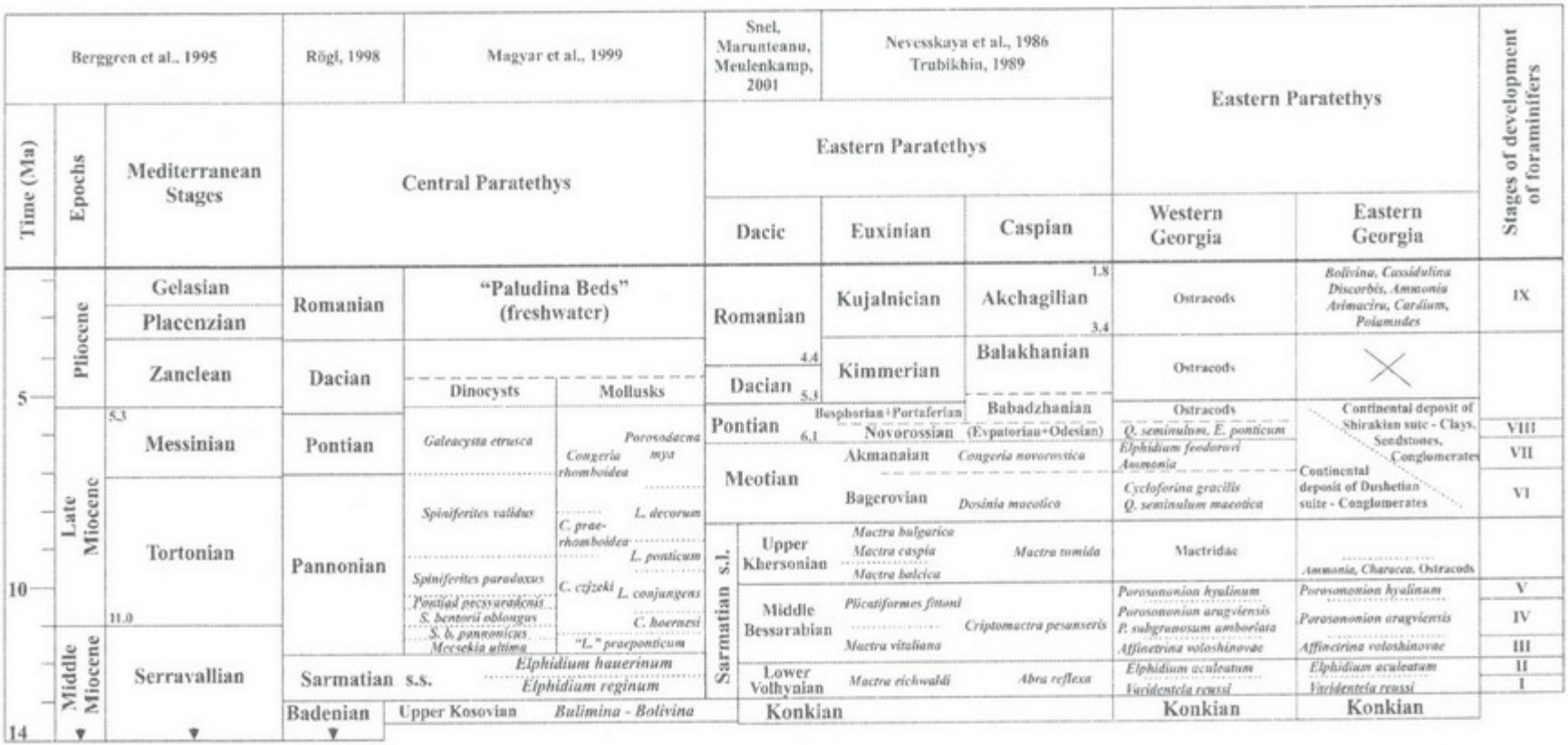


Fig. 2. The Correlation of states of development of foraminifers and bioevents with standard stratigraphical units of the Upper Cenozoic deposits of Georgia

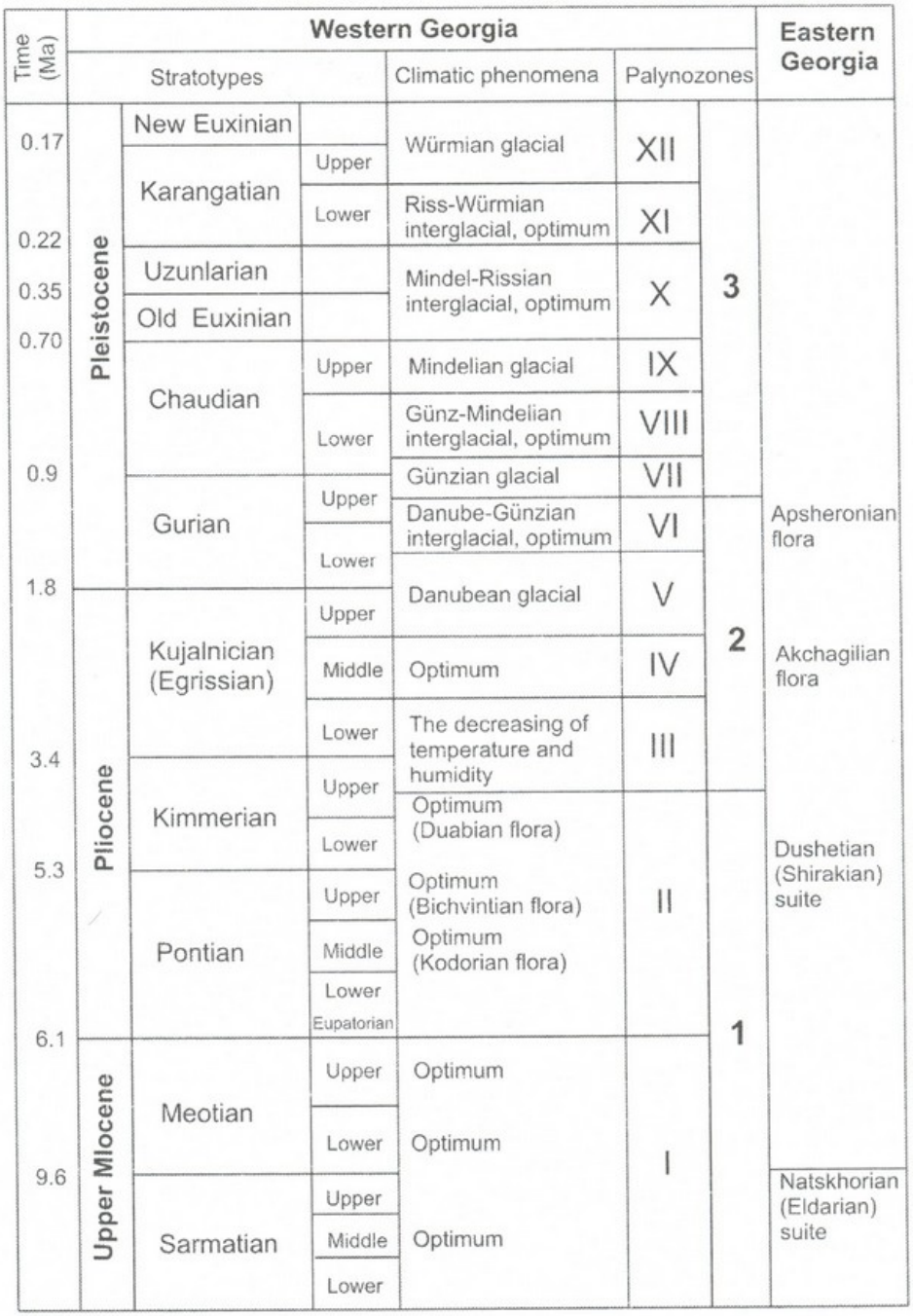


Fig.3. The correlation of palynozones (stages) with standard stratigraphical units of the Upper Cenozoic deposits of Georgia (the absolute ages are given by data of Borzenkova, 1992 and Taktakishvili, 1999).

Consequently, after the Sarmatian, the vegetation in Western and in Eastern Georgia developed independently under the influence of different climate conditions. This phenomenon can be connected with orogenic movements, which led to important paleogeographical changes: draining of the Transcaucasus strait, division of the territory of Georgia into two regions and isolation of Colchis. After the Sarmatian, marine conditions were preserved only in Western Georgia.

The second turning-point occurred in the upper part of the Kimmerian and in the lower part of the Kujalnician (**stage III**). This interval of time began and finished by the domination of

pine, the area of which periodically prevailed over polydominant forest. This again indicates an decrease of humidity and probably also temperature (Fig.6). Several authors [Milanovsky, 1968; Tsagareli, Astakhov, 1971] are of the opinion that at approximately this time the lowering of relief of the Greater and Lesser Caucasus took place. This could be the reason of disturbance of isolation of Colchis and changes in climatic conditions that resulted in mass extinction of subtropical plants, which lost their coenotical value. The polydominant forest of the next stage IV (Middle Kujalnician) was composed mainly by deciduous trees, but along with them the relics of ancient floras continue to exist.

The third turning-point was the time probably corresponding to the end of the Kujalnician and the beginning of the Gurian (boundary between **stages IV** and **V**), when climate changed in Western Georgia. The main trend was the increase of humidity and periodical changes of temperature connected to glacial and interglacial periods. Stage V corresponds to the first strong cooling, which is correlated with the Danubean glacial. During this time, the whole territory of Western Georgia was covered by dark-conifer forests (Fig.6).

The following time – the upper part of Early Gurian and lower part of the Late Gurian (**stage VI**) can be correlated with the Danubean-Günzian interglacial (Fig.6). It was the last period of blossoming of rich polydominant forest, the area of which was often much bigger than the area of other vegetation types.

Later in **stages VII, VIII**, and **IX** (the end of the Late Gurian and the whole Chaudian) began the process of splitting of polydominant forest into separate formations. This was related to new orogenic movements, which transformed the Greater and Lesser Caucasus into huge mountain structures, close to the outline of today's mountain ranges (Kogoshvili, 1977; Milanovsky, 1977; Tsagareli, 1980). The Colchis again became an isolated region with dismembered relief, with favorable conditions for temperate and warm-temperate plants. They were distributed according to the altitude and produced a new structure of vegetation. Three forest belts under different climatic conditions were formed: an upper belt with dark-conifer forest, a middle belt occupied by monodominant beech forest, and a lower belt with mixed forest where more thermophile plants concentrated (Fig. 7). This structure was close to the modern one, although the composition of forest was much richer.

Thus, after the third turning-point during **stages V-XII** (Fig 6, 7) covering the time from the Late Kujalnician till the end of the Pleistocene, the main climatic factor influencing the dynamics of vegetation was temperature. Its fluctuations depended on the alternation of glacial and interglacial periods, from the Danubean till the Würmian (Fig. 3). Humidity changed only insignificantly.

The analysis of the whole palynological material shows that radical changes of phytocenosis were connected with decreasing humidity that took place in first part of the Late Cenozoic. During this time, important changes occurred in the composition of flora and in the character of vegetation both in Eastern and Western Georgia. All these phenomena probably were connected with changes in relief of the Caucasus during the Late Miocene and Early Pliocene.

The process of development of flora and vegetation in Western Georgia during the Late Pliocene and Pleistocene was of somewhat different character. During this time, the Greater and Lesser Caucasus had attained nearly the altitude of today's mountains, stimulating the forming of vegetation with a structure similar to the recent one. In the isolated ecosystem of Colchis, the development of vegetation mainly under the influence of fluctuation of temperature was less important, which led only to the displacement of boundaries between the vegetation types. Concerning the evolution of flora, it was mainly marked by the extinction of separate elements that continued until the end of the Pleistocene.

In summary, the history of development of vegetation during the Late Cenozoic can be divided into three main intervals (Fig. 3).

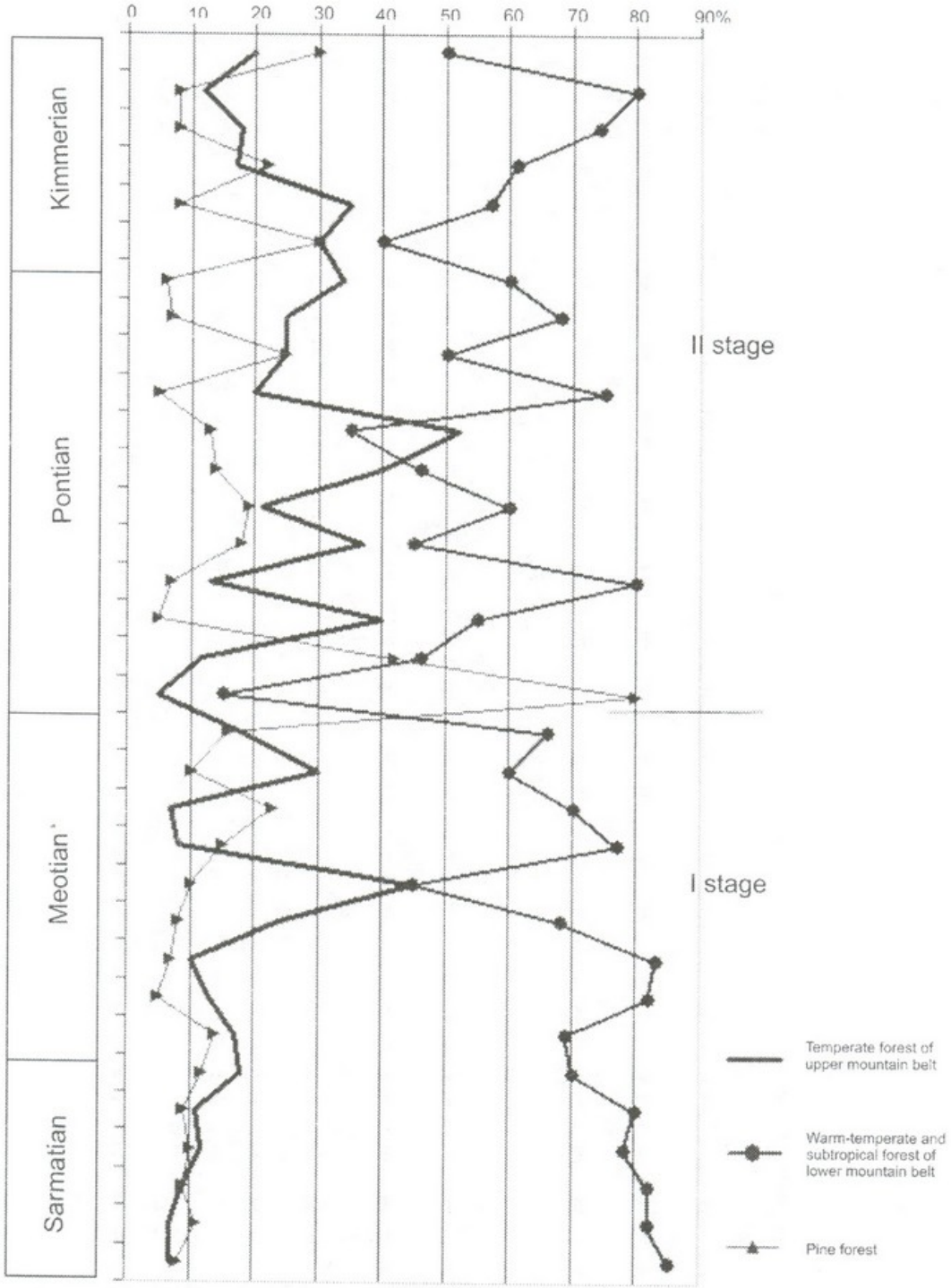


Fig.4. The changes of area of main forest types of Western Georgia during I-II stages.

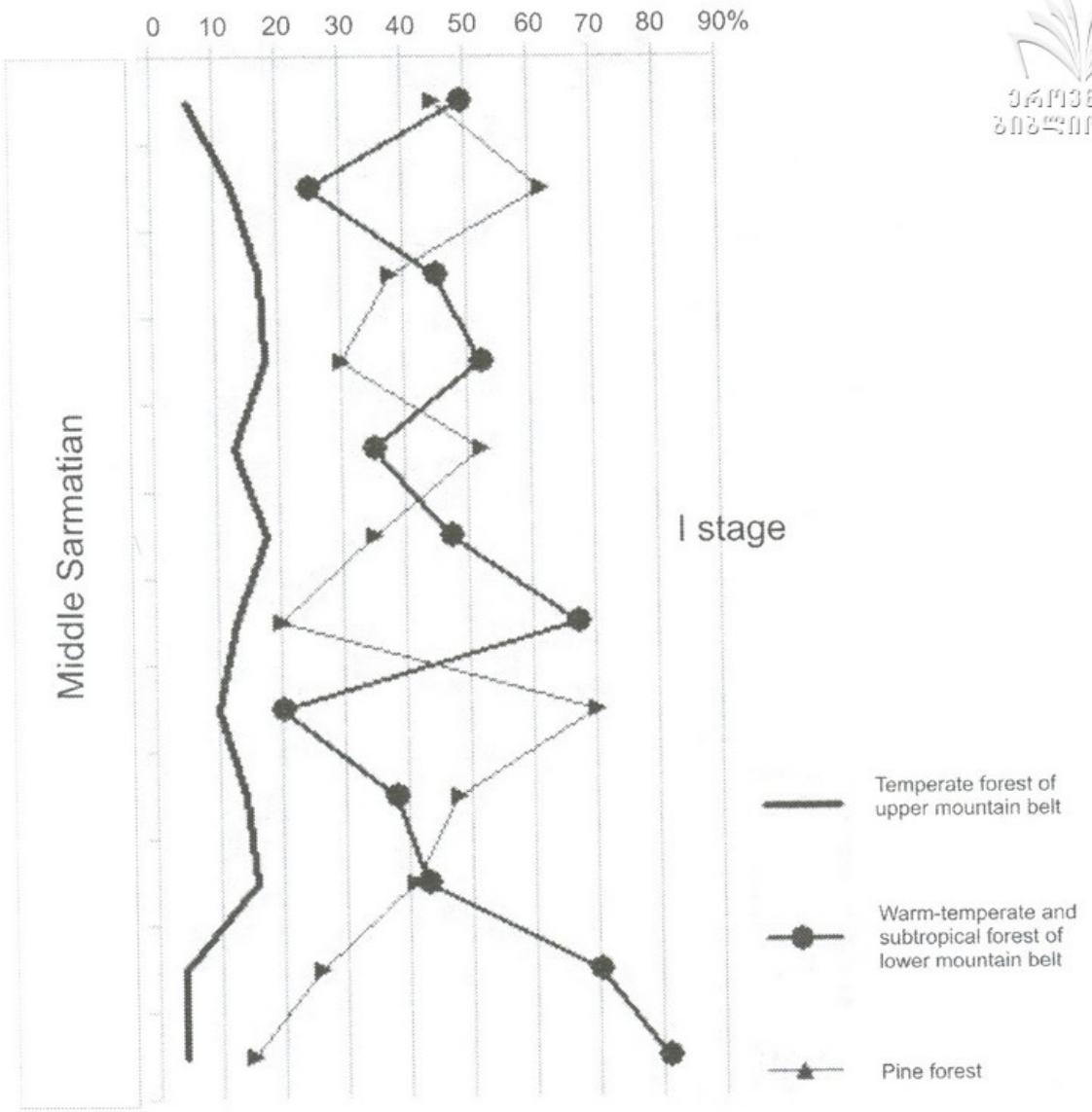


Fig.5. The changes of area of main forest types of Eastern Georgia during I stage.

The first one is characterized by the domination of subtropical forest. On the territory of Eastern Georgia, this formation existed only in the Early and Middle Sarmatian. However, in Western Georgia, it occurred during the Sarmatian, Meotian, Pontian, and the main part of the Kimmerian (**stages I, II**). The identification of the following intervals is based only on material from Western Georgia.

The second interval covers the transitional period (**stages III-VI**), when the vegetation preserved old features but also obtained new ones. At this time began the division of leaf-bearing and conifer polydominant forests as separate coenoses and their distribution according to relief.

The third interval (**stages VII-XII**) was the time of predominance of vegetation, the main character of which is preserved till now in Western Georgia. During this interval the extinction of Tertiary relics, was completed.

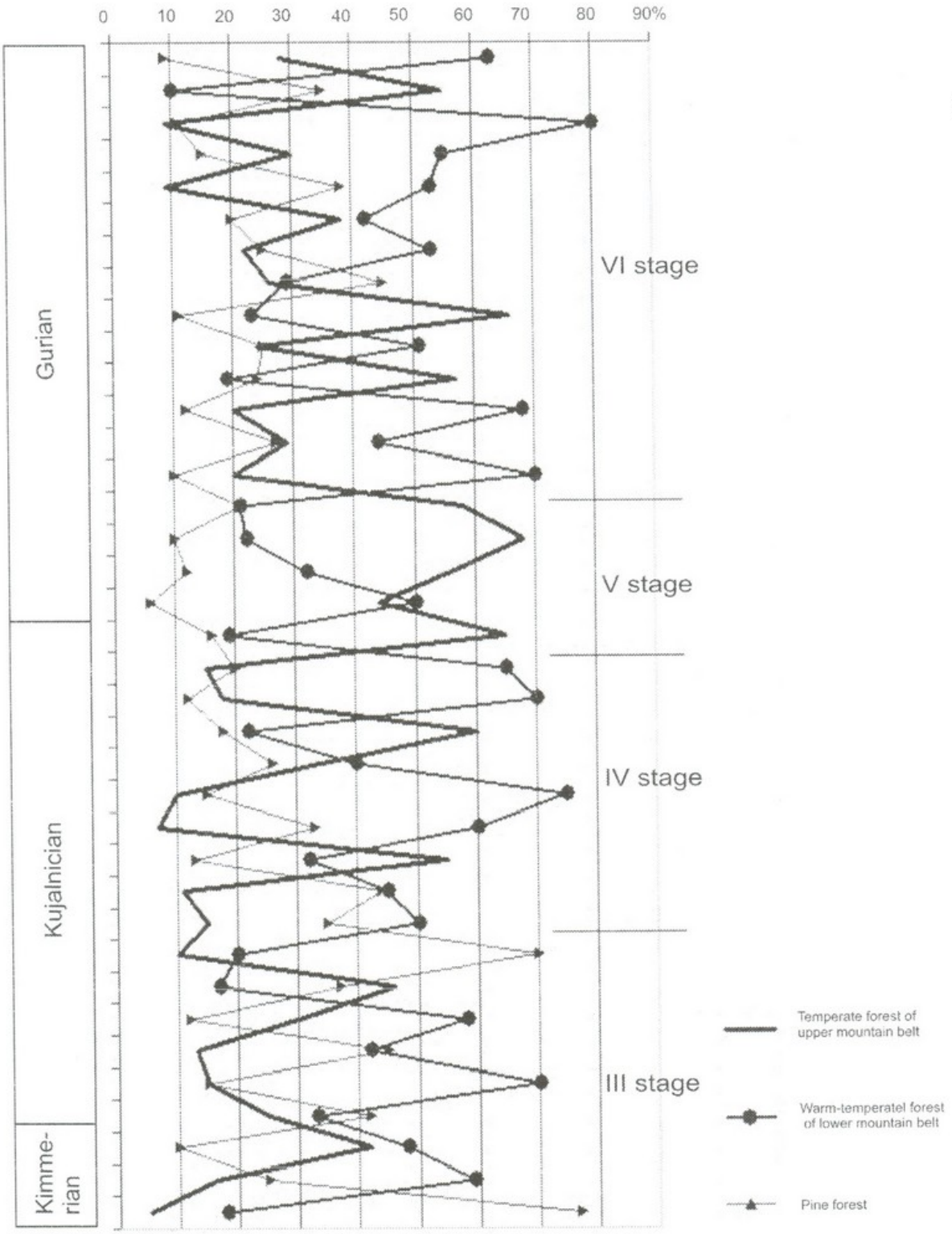


Fig.6. The changes of area of main forest types of Western Georgia during III-VI stages.

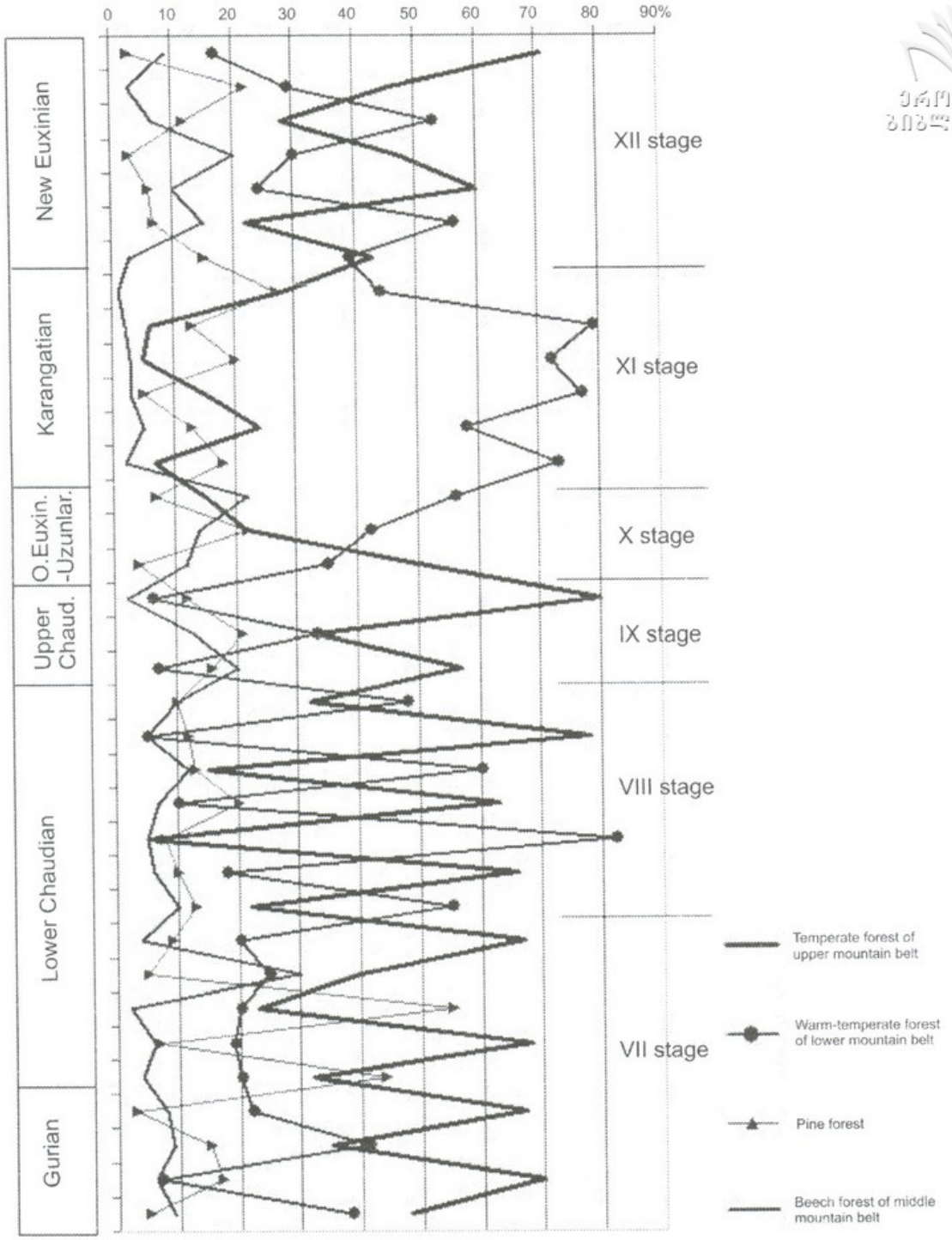


Fig.7. The changes of area of main forest types of Western Georgia during VII-XII stages.

Conclusion

During the Late Cenozoic several turning-points in the evolution of marine and terrestrial biocoenoses in Georgia can be distinguished. The relation of some of them to one and the same statigraphical boundaries permits to presume some significant changes in the environment, which led to these bioevents. One such boundary is the transition between the Middle and Late Sarmatian, when on the territory of the Caucasus an important paleogeographical transformation took place.

The Transcaucasian intermontane depression became dry land with two bays, Kurian and Rionian. In the East, except for some regions, continental deposits started to form, while in the West, along the Rionian Bay, the uninterrupted accumulation of the marine sediments continued.

By the end of the Middle Sarmatian, significant changes are observed in the composition of the marine biocoenosis, owing to the further freshening of the basin. The rich and diverse fauna of benthonic foraminifers and mollusks gradually declined and by the end of the Middle Sarmatian almost entirely died out. Out of the benthonic foraminifers, only *Ammonia* and a few *Porosononion* survived. Among the mollusks, only *Mactra* was preserved, which was predominant in the Late Sarmatian basin.

The end of the Middle Samatian was a turning-point in the development of terrestrial flora as well. It was probably connected with orogenic movements, due to which Georgia turned into a high mountainous country divided into two major regions – Western and Eastern. The territory adjoining the Black Sea was separated into an isolated region, the Colchian refuge, where a relatively mild climate prevailed, which was favorable for the development of rich forests. In Eastern Georgia the process of xerophitization was over and domination of coenoses of open places began. Consequently, after the end of the Sarmatian, quite different climatic conditions dominated in Eastern and Western Georgia. This paleogeographical situation is preserved until today.

The second common turning-point in the evolution of terrestrial and marine biocoenoses is the boundary between Miocene and Pliocene – the Eupatorian horizon, when the impoverishment in composition of marine organisms and of vegetation took place, probably connected to a worsening of climatic conditions.

Nevertheless, after the Eupatorian and during the whole Pontian and most of the Kimmerian on the territory of Western Georgia, rich subtropical forests of a warm-temperate climate continued to exist. They were restricted to the habitat where the required ecological conditions were preserved. By the end of the Kimmerian, the subtropical formations disappeared and the warm periods of the Kujalnician and Gurian were characterized by the domination of polydominant deciduous and conifer forests. At the end of the Gurian these began to divide into separate coenoses, in structure close to modern ones.

The development of flora and vegetation in Georgia during the Late Cenozoic was controlled by biotic and abiotic factors. The tectonic movements and climate were important among the latter. Climatic fluctuations became more pronounced in the Pliocene and especially in the Pleistocene, in connection with glacial and interglacial periods in the Caucasus.

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საქართველოს გვიანკაინოზოურში განვითარებული ბიომოვლენები ფორამინიფერებისა და პალინომორფების მონაცემებით.

შატილოვა ი.[1], მაისურაძე ლ.[1], ქოიავა კ.[2], მჭედლიშვილი ნ.[1], რუხაძე ლ.[1], სპეზაფერი ს.[3], სტრასერი ი.[3]

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(მიღებულია 15.12.2008)

რეზიუმე

გვიანკაინოზოური ნალექებიდან შესწავლილია ფორამინიფერები და პალინომორფები. აღმოსავლეთ და დასავლეთ საქართველოს ტერიტორიაზე სულ გაანალიზებულია 45-ზე მეტი ჭრილიდან მოპოვებული მასალა. ფორამინიფერები შესწავლილია სარმატული და მეოტური ნალექებიდან და გამოვლენილია მათი ისტორიული განვითარების ეტაპები. ლანდშაფტურ-ფიტოცენოლოგიური მეთოდის გამოყენებით დადგენილია გვიანკაინოზოური მცენარეულობის ევოლუციის ძირითადი ეტაპები, რომლებიც დაკავშირებულია კლიმატურ ფლუქტუაციებთან. ზღვიური და ხმელეთის ბიოცენოზების განვითარების ისტორიაში გამოვლენილია რამდენიმე ძირითადი გარდატეხის მომენტი, რომლებიც შეესაბამება პალეოგეოგრაფიული პირობების ცვლილებებს კავკასიის ტერიტორიაზე გვიანკაინოზოურის განმავლობაში.

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EXCRACTION AND PURIFICATION OF ENZYME ARGINASE AND STUDY OF SOME PHYSICAL-CHEMICAL FEATURES OF ITS ISOFORMS IN THE CESTODE *PIRAMICOCEPHALUS PHOCARUM* PLEROCERCOID

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Abstract

Purification of the arginase extracted from the cestode of *Piramicocephalus phocarum* was carried out by the gel-filtration and ion exchange chromatography methods. The enzyme appeared to have three peaks of activity, which corresponds to the existence of three isoforms and is also detected by the specific staining of gel electrophoresis. Molecular masses of single isoforms on a Sephadex G-200 column were established by gel-filtration and pH optimums.

Key words: arginase, isoforms, gel-filtration, *Piramicocephalus phocarum*

Introduction

Cestodes are parasitic worms and they cause various diseases of human, animals and plants. Cestodes are characterized by difficult development cycle.

During the whole development cycle they change their environment many times and undergo influence of such agents as nutrition, immune system of the host, oxygen, temperature, etc. Hence, it is interesting to study those mechanisms which determine adaptation of both, adult and larval forms of the parasite at the cellular level.

Intercellular biochemical and molecular mechanisms underlie the performance of adaptive reactions at the cellular level.

Enzymes play a great role in the biochemical mechanisms of adaptation of helminthes. Among these enzymes arginase often occurs in cestodes. Therefore the aim of our study was extraction – purification of enzyme arginase and study of some physical-chemical features of the obtained isoforms (molecular mass and pH optimum).

Material and Methods

Piramicocephalus phocarum Plerocercoid was selected as an object of study.

To purify arginase gel-filtration and ion exchange chromatography methods were used.

The investigated homogenate was obtained as a result of homogenization of *Piromicocephalus phocarum* plerocercoid. Homogenization was carried out in buffer (1 mM hydrochloric acid, 1 mM manganum chloride, 0.5% triton X- 100, pH 7.3). After centrifugation of

the homogenate isolation of protein fractions from the obtained supernatant was carried out using 30-60% saturated ammonium sulfate. To isolate ammonium sulphate and proteins of small molecular mass from the obtained fractions samples were deposited on a Sephadex G-100 column. Fractions having arginase activity were separated from the fractions obtained by gel-filtration and then collected. For further purification of the enzyme collected fractions were deposited on chromatography column (2.5X12cm) of DEAE cellulose equilibrated with buffer (5ml Tris. HCI, iml manganum chloride, 10% glycerine, pH 8.3). Enzyme elution was carried out with a gradient of increasing sodium chloride concentration starting from 0.1 M to 0.3 M with the buffer of the same composition at a rate of 20 ml/h.

Determination of protein concentration and arginase activity were carried out in 1 ml fractions obtained as a result of elution.

Determination of enzyme activity was carried out by the combined method [Khramov V.A., Galaev J.V. 1969]. The gel was adjusted in specific incubating area consisting arginine (850ml), urease (2mg/ml phosphate buffer pH 6.2), 0.6 ml 0,1M ditiotreitol and 1.3% tetrazolium. Incubation was carried out in thermostat at 37°C for 30-45 minutes [Farooqui et al., 1978].

Determination of protein concentration was carried out by the Lowry method (Lowry et al., 1951)

Isoforms of enzyme arginase were studied by the method of disc-electrophoresis in 7.5% polyacrylamide gel [Maurer, 1971].

Results and Discussion

Results of arginase purification are given in Table 1. As is seen relative enzyme activity after ion exchange chromatography is 1875 urea/min per mg protein and it approximately 480 times exceeds homogenate activity.

The profile of the elution from the ion exchange column is given on the Fig. 1. The figure detects that the elution profile coincides with the profile of arginase activity and three peaks of arginase activity were noticed. It points out to the existence of three different arginase isoforms and is verified by electrophoregram in polyacrylamide gel (Fig.2).

Table 1. The scheme of arginase purification

Stage of purification	Volume, ml	Total activity, unit	Total protein, mg	Yield activity, %	Specific activity, unit/ mg protein
Homogenate	110	30 000	10 000	100	3,9
Supernatant	90	29 000	7 000	96	4,1
40-60% saturation ammonium sulfate fraction	30	20 000	400	66	5
Fraction eluded from the Sephadex G-100 column	25	18 000	70	60	257
Fraction eluded from DEAE cellulose column	20	15 000	8	50	1875

As it is shown on Fig.2 isoformic spectrum of arginase is represented by three isoforms that proves the results obtained by ion exchange chromatography, where three peaks of arginase activity are demonstrated. Multiple forms of arginase were also detected in many representatives of vertebrate animals [Que et al., 1998].

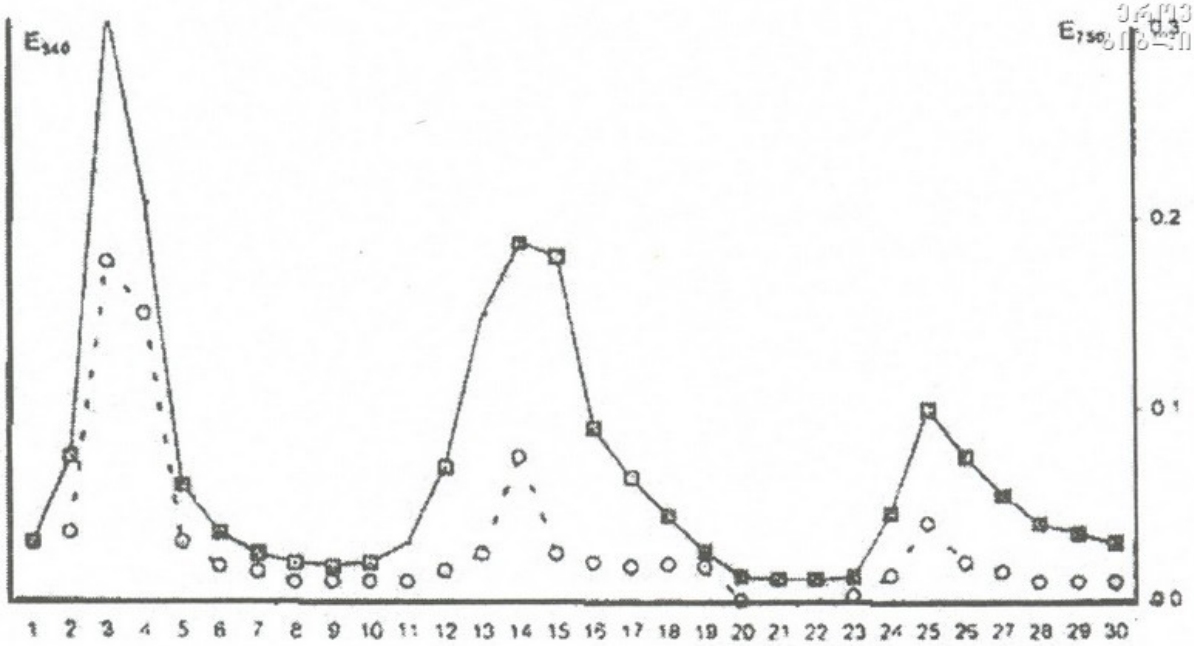

Fig. 1. Elution profile on diethylaminoethyl cellulose column after ion-exchange chromatography

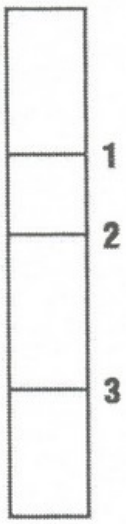

Fig. 2. Spectrum of arginase isoforms in 7.5% polyacrilamyde gel.

On the next stage molecular masses and pH optimums of the obtained isoforms were established. The results are demonstrated in Fig.3, 4, 5, 6.

Molecular mass of three isoforms were determined by gel-filtration on a Sephadex G-200 column, previously calibrated using molecular mass markers. Molecular mass of all three isoforms determined by its elution volume (the elution volume was established by determination of protein concentration and enzyme activity in eluted fractions), using a special formula and also relying on analogical characters of markers of molecular masses, are approximately equal (approximately 100 kD).

Study of all three isoforms activity in buffers with different pH showed that the first isoform reveals maximum activity at pH 9.3 (Fig.4), the second isoform - at pH 8.0 (Fig.5), the third – at pH 7.5 (Fig.6).

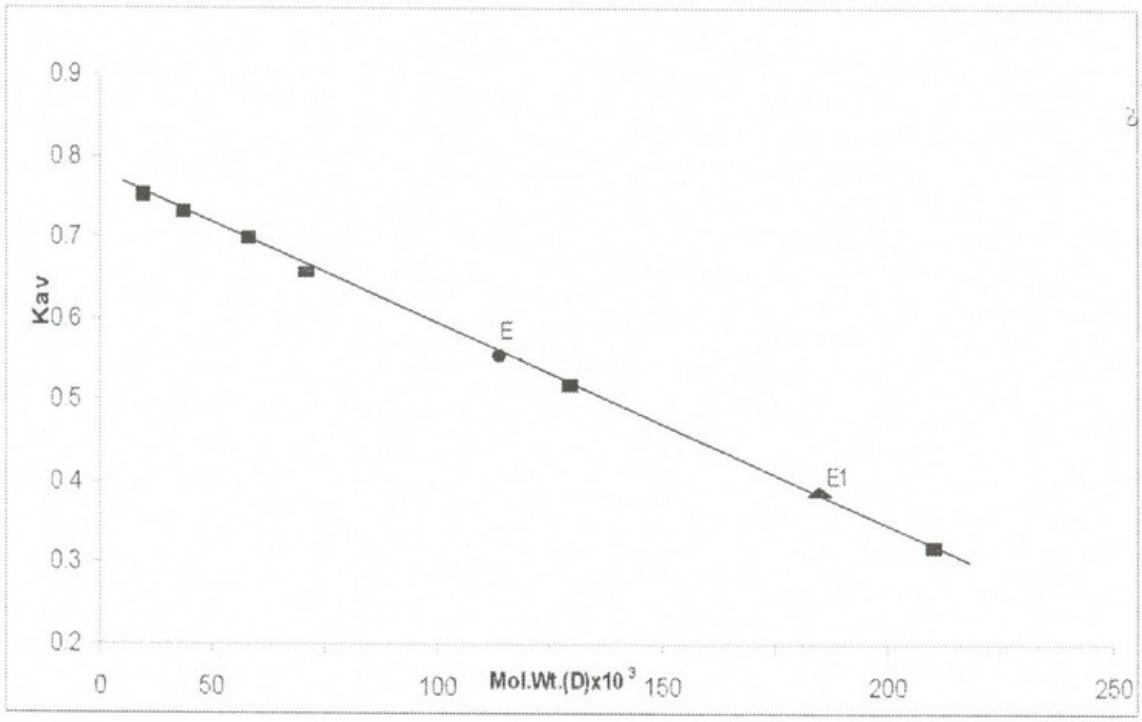

Fig. 3. Determination of molecular mass by gel-filtration on a G-200 column

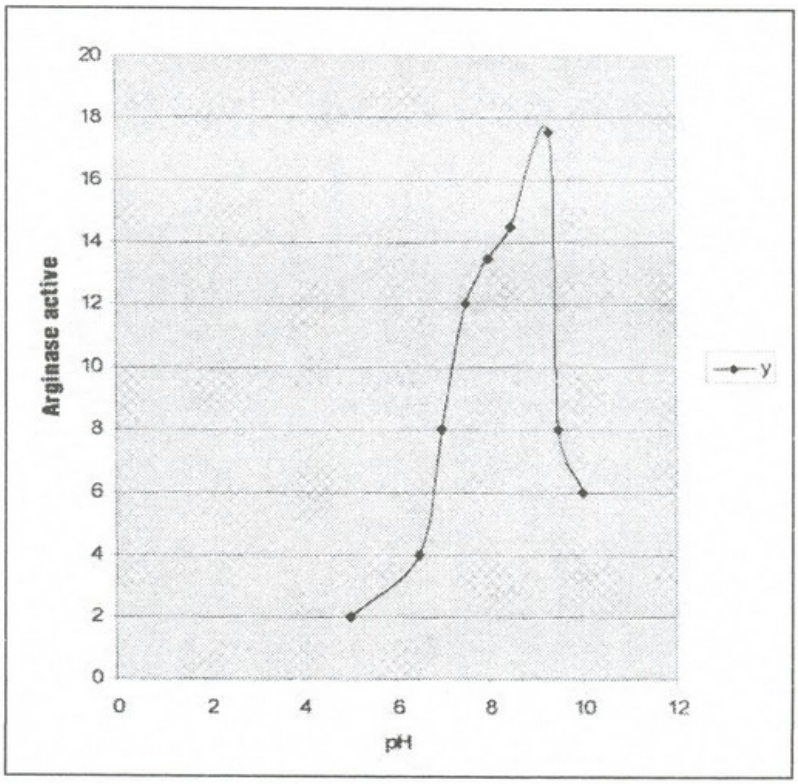

Fig. 4. Influence of pH on I isoform of arginase

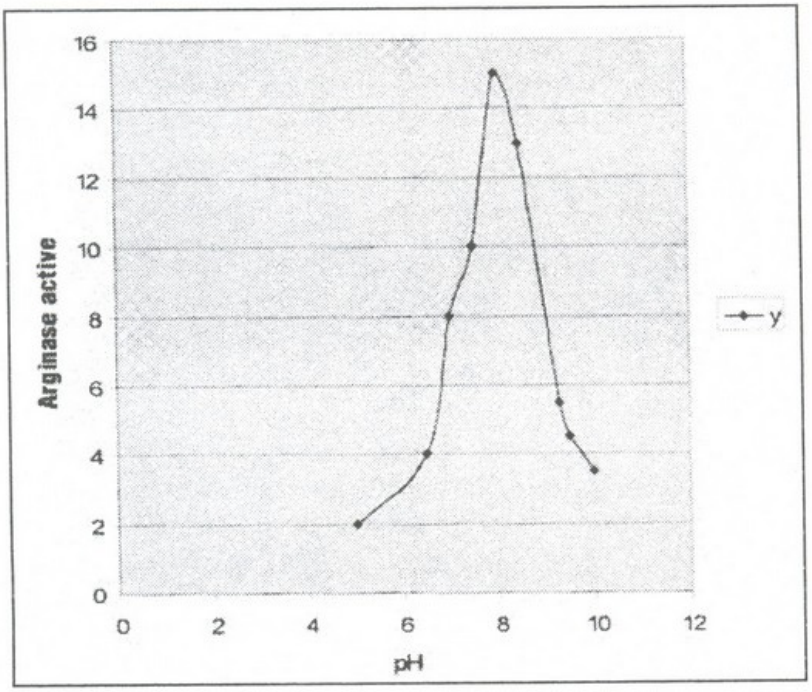

Fig. 5. Influence of pH on II isoform of arginase

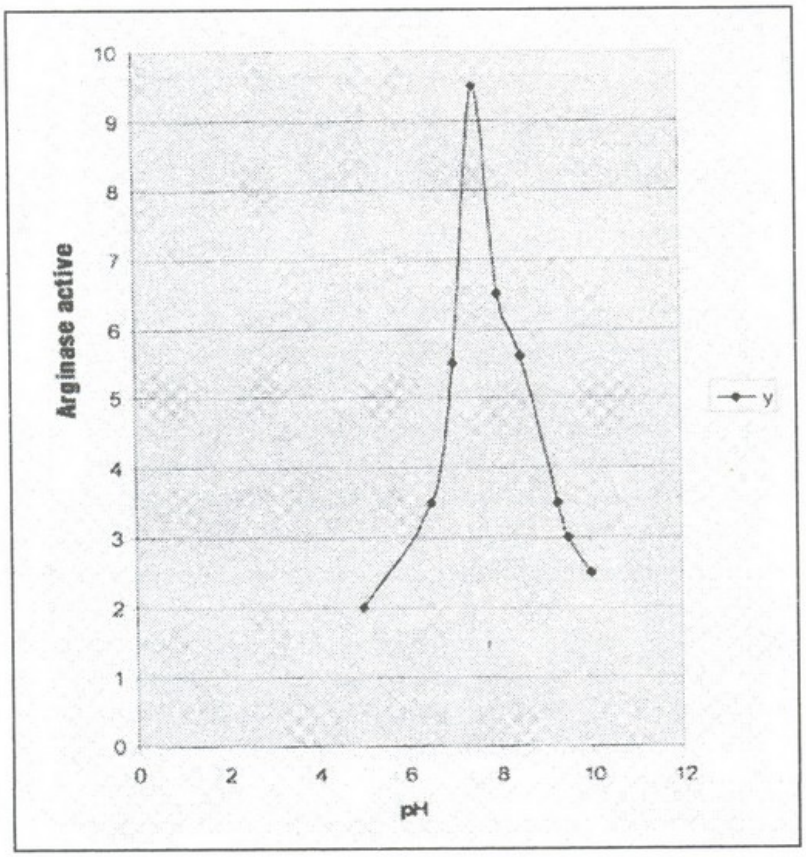

Fig. 6. Influence of pH on III isoform of arginase

Therefore arginase isoforms have different pH optimums. It is also interesting that pH optimum of arginase isoforms in mammals varies within the limits of pH 9.5-10.5 [Christopher et

al., 1994; Kuhn et al., 1991]. Such difference of activity on different pH could possibly point out that ionized groups must influence on catalyzed centre of the enzyme.

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არგინაზას გამოყოფა-გაწმენდა და მისი ზოგიერთი ფიზიკურ-ქიმიური მახასიათებლების შესწავლა ცესტოდა ***Piramicocephalus phocarum*** – ის კლეროცერკოიდში.

მამაცაშვილი ა., მელაშვილი ნ.

ზოოლოგიის ინსტიტუტი

(მიღებულია 11.12.2008)

რეზიუმე

გელ-ფილტრაციის და იონცვლადი ქრომატოგრაფიის მეთოდებით მოხდა *Piramicocephalus phocarum*-ის ცესტოდადან გამოყოფილი არგინაზას გაწმენდა. ფერმენტს აღმოაჩნდა აქტივობის სამი პიკი, რაც შეესაბამება მასში სამი იზოფორმის არსებობას, და რაც ასევე დადასტურდა ელექტროფორეზით გელის სპეციფიკური შეღებვისას. დადგენილ იქნა ცალკეული იზოფორმების მოლეკულური მასები სეფადექს G-200-ის სვეტზე გელ-ფილტრაციით და pH ოპტიმუმები.

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DETERMINATION OF DB1 LECTIN-BINDING SITES IN *HELICOVERPA ARMIGERA* MIDGUT TISSUES

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Abstract

The binding of DB1 lectin to midgut epithelia of *Helicoverpa armigera* was examined by ABC staining. DB1 strongly bound to larval brush border and basement membrane. The results propose that antinutritive effects of DB1 may be attributed to specific binding and subsequent toxic effects in the midgut of insects.

Key words: *Helicoverpa armigera*, midgut, lectin.

Introduction

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is a very destructive polyphagous pest occurring on cotton, tomato, chickpea, pigeon-pea, chilli, maize, sorghum and many other crops, inflicting substantial crop losses every year. Exogenous chemical means to counteract *H. armigera* attack have become less feasible, mainly due to the development of pesticide resistance and inherent possible environmental hazards [Ramasubramaniam, T. and Regupathy, A. 2004].

With the exception of some enzymes, e.g. some types of chitinases, glucanases, glycosidases, lectins are only plant proteins that are capable of recognizing and binding glycoconjugates on exoskeleton of invertebrates or specific sites exposed along the intestinal tract. Such interaction is considered to be prerequisite for insecticidal action [Rudiger, H. and Gabius, H.J. 2001].

We showed DB1 inhibited *H. armigera* larval development when incorporated into artificial diet of insects [Gaidamashvili, M., et al., 2008a]. DB1 had high sequence similarity to GNA (*Galanthus nivalis* bulb lectin), whose anti-nutritive effects to insect pests is well documented [Gaidamashvili, M., et al., 2008b].

In the present paper we analyzed binding of the mannose-binding DB1 lectin to larval gut epithelial cells and determined lectin-binding sites in *H. armigera* midgut tissues.

Materials and Methods

DB1 lectin and larval cultures of *H. armigera* were prepared as described [Gaidamashvili M., et al., 2008a]. Biotinylated lectins were prepared as follows: DB1 was dissolved in 0.1 M sodium bicarbonate buffer (pH 8.5) and reacted with 0.5 mg of biotin *N*-hydroxysuccinimide ester (Sigma, St. Louis, USA) (in 10 µl of dimethylsulfoxide) at room

temperature for 4 h. The reaction mixture was then dialyzed against Dulbecco PBS. Solutions were aliquoted and stored at -20°C as a stock solution of biotinylated lectin (B-DB1).

Insects were fixed by 4% paraformaldehyde in CMF-PBS for 48 h at room temperature. Fixed specimen was dehydrated in a graded series of ethanol, allowing 12 h for each step. Then the specimen was infiltrated and embedded with toluene-paraffin solution (1:1) over night followed by paraffin for 3x 2 h at 56-58°C. Sections of 4 μm thickness were cut on a microtome.

Dewaxed and hydrated paraffin sections of insects were washed with running tap water for 5 min. They were incubated with B-DB1 or B-GNA (EY Labratories, San Mateo, USA) for 30 min. The slides were washed with PBS for 10 min. The sections were incubated with avidin-biotinylated peroxidase complex (ABC) reagent (VECTASTAIN® Elite ABC Reagent, Vector Laboratories, CA, USA) for 30 min and washed with PBS for 10 min. The sections were incubated with 3,3'-diaminobenzine (DAB) and H_2O_2 as substrates in PBS until desired stain intensity developed.

Results and Discussion

As it is demonstrated in Fig. 1A, DB1 binds to the midgut region of larval tissues. Binding was predominantly observed in the basement membrane of midgut, and was developed more intensively in compare to brush border region. The binding pattern was much similar to that of GNA (Fig. 1B), which preferably binds to the structures containing α-1,3-mannose residues. Similarity in binding sites of DB1 and GNA to midgit region was largely expected due to their resemblance in sugar specificity. Snowdrop (*Galanthus nivalis*) bulb lectin GNA is the first lectin reported from this mannose-binding lectin family having well defined insecticidal properties to a range of economically important pests [Fitches, E., et al., 2001]. DB1 had high homology to GNA, especially to its carbohydrate-binding site [Gaidamashvili, M., et al., 2008b].

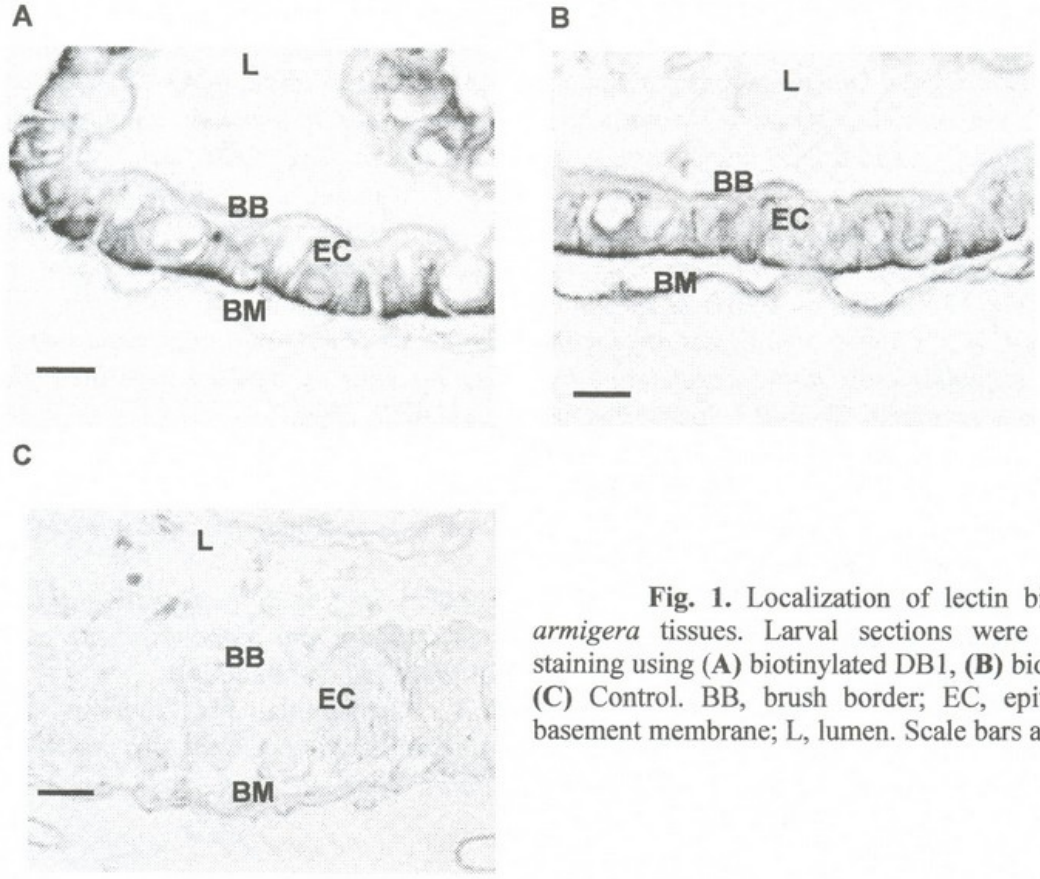

Fig. 1. Localization of lectin binding sites in *H. armigera* tissues. Larval sections were stained by ABC staining using **(A)** biotinylated DB1, **(B)** biotinylated GNA, or **(C)** Control. BB, brush border; EC, epithelial cells; BM, basement membrane; L, lumen. Scale bars are 50 μm.

Binding of lectins to the peritrophic membrane and subsequent toxic effects were reported for a number of insects [Habibi, J., et al., 2000]. Zhu-Salzman et al. (1998) have provided the dependency of insecticidal activity upon lectin binding to the midgut epithelium or peritrophic membrane. The deleterious effects of such binding involve the increase of permeability of peritropic membrane and restriction of bi-directional movement of nutrients and digestive enzymes across membrane pores. Binding lectins to the peritrophic membrane appears to be necessary condition for insecticidal activity, though the effects may not always be attributed with direct damage of gut epithelia [Sauvion N., et al., 2004]. On the contrary, PHA, in particular the isolectin E_4, apparently toxic to potato leafhopper (*Eempoaca fabae*), was shown binds to the midgut epithelial cells and leads to severe disorganization and finally, to occlusion of the lumen [Habibi, J., et al., 1998]. It is very likely, that perceptible damage of the gut integrity is largely depending on the avidity of lectin binding as well as to long-term exposure to its toxic dose.

Helicoverpa species of Lepidoptera family are serious pests on various agricultural crops including Solanaceae, Poaceae, Fabaceae, Cucurbitaceae. Dioscoreaceae species are also undergoing to attack, but losses are rather moderate in compare to other crops. DB1 existed in yam tubers at significant amounts (20% of total protein content), where it is accumulated as a storage protein. Preferential binding of GNA-like protein to midgut structures of insect pests and its antinutritive effects to *H. armigera* larvae might be an additional argument for proposed protective role of DB1.

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DB1 ლექტინის დამკავშირებელი უბნების განსაზღვრა *Helicoverpa armigera* ნაწლავის ჭსოვილებში

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რეზიუმე

ბიოტინილირებული ლექტინის გამოყენებით შესწავლილია *Helicoverpa armigera* ნაწლავში DB1 ლექტინის დაკავშირება ეპითელურ სტრუქტურებთან. DB1 მტკიცედ უკავშირდებოდა ლარვას შუა-ნაწლავის ჯაგრისოვან სარტყელს და ბაზალურ მემბრანას. შედეგები მიუთითებს, რომ DB1 ანტი-კვებითი ეფექტები შეიძლება განპირობებული იყოს ლექტინის სპეციფიკური დაკავშირებით და თანამიმდევრული ტოქსიკური ეფექტებით მწერების საჭმლის მომნელებელ ტრაქტში.

ინსტრუქცია ავტორთათვის

სამეცნიერო ნაშრომი გამოიცემა ინგლისურ ენაზე, მას უნდა დაერთოს რეზიუმე ინგლისურ და ქართულ ენაზე, სამეცნიერო მიმართულება, სათაური, ავტორთა გვარები და მათი სამუშაო დაწესებულების დასახელება, საკვანძო სიტყვათა მოკლე (4-6) სია.

წერილის მოცულობა არ უნდა იყოს 5 გვერდზე ნაკლები და 12 გვერდზე მეტი. წერილი უნდა გაფორმდეს შემდეგი რუბრიკაციით: შესავალი და მიზნები (Introduction), მასალა და მეთოდები (Materials and Methods), შედეგები და მათი განხილვა (Results and Discussion), დამოწმებული ლიტერატურა. უკანასკნელი უნდა იყოს დალაგებული ანბანის მიხედვით, ხოლო ტექსტში წყაროების მითითება უნდა ხდებოდეს ფრჩხილებში ჩასმული ავტორის გვარითა და წლით [Lernmark, Hagglof 1981].

მითითებული ლიტერატურა წარმოდგენილი უნდა იყოს შემდეგნაირად:

ჟურნალის შემთხვევაში

Carvalho C., Pereira H., Pina C. *Chromosomal G-dark bands determine the spatial organization of centromeric heterochromatin in nucleus.* Mol. Biol. Cell, **12**, 5, 3563-3572, 2001.

წიგნის შემთხვევაში

Kuhn T.S. *The structure of scientific revolutions.* Chicago, IL, Chicago Press, 2000.

Brush S. *Flowing waters or teeming crowds.* In: Mental Models. D. Gentner (Ed.), Chicago IL., Chicago Press, 865-900, 2001.

მასალა რედაქციაში წარმოდგენილი უნდა იყოს ქაღალდზე ამობეჭდილი და დისკეტით (ან CD-ით). წერილი ერთი ფაილით უნდა იყოს შენახული (ცალკე ფაილად შეიძლება ილუსტრაციების წარმოდგენა), ხოლო ფაილის სახელწოდება წერილის პირველი ავტორის გვარს უნდა ატარებდეს.

ქართული ტექსტისთვის ოპტიმალური ფონტებია AcadNusx და AcadMtavr, ინგლისური ტექსტებისთვის - Times New Roman. შრიფტის ზომა - 12 პუნქტი, ინტერვალი - 1,5. ცხრილებში დასაშვებია უფრო მცირე ზომის შრიფტები. წერილი უნდა დაიბეჭდოს A4 ფორმატით, ზევით და ქვევით - 2,5 სმ., მარცხნივ – 3 სმ. და მარჯვნივ – 2სმ. დაშორებით. ცხრილები, გრაფიკები და დიაგრამები (მხოლოდ შავ-თეთრი) შესაძლებელია დამზადდეს როგორც Microsoft Word-ში, ისე Excel-ში, ფოტოსურათები მიიღება აგრეთვე ორიგინალების (არაელექტრონული) სახითაც.

ჟურნალის გამოცემა ავტორთა ხარჯებით ხორციელდება. თანხა რედაქციაში უნდა შემოვიდეს ნაშრომზე დადებითი რეცენზიის მიღებისთანავე. ნაშრომის რეცენზირება ანონიმურია და ავტორს აქვს უფლება მიიღოს ან არ მიიღოს რეცენზენტის შენიშვნები. უკანასკნელ შემთხვევაში ნაშრომი, დამატებით გაეგზავნება სარედაქციო საბჭოს ერთ-ერთ წევრს. მეორე უარყოფითი დასკვნის შემთხვევაში, ნაშრომი არ გამოქვეყნდება.

ნაშრომის ჩაბარება შეიძლება სამუშაო დღეებში, 12-დან 16 საათამდე, შემდეგ მისამართზე: თბილისი, რუსთაველის გამზირი 52, საქართველოს მეცნიერებათა აკადემია, ბიოლოგიის განყოფილება, IV სართული, 429 ოთახი, ტელ: 93-58-92, პასუხისმგებელი მდივანი – მაია გრიგოლავა.