Analysis of microbial phospholipids in processes of biomonitoring of soil condition

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Abstract: *The purpose of this work was to assess the possibility of applying the method of phospholipid fatty acid analysis in the composition of lipids of cell membranes of soil microorganisms for monitoring the state of the soil in the process of recovery. The experimental part consisted in a determination of phospholipid fatty acids (FFA), including extraction of phospholipids with organic solvents, extraction of the lipid fraction using polar silicate columns, preparation of methyl esters of fatty acids by alkaline methanolysis and analysis of the content of FFA with gas chromatography. The identification of microorganisms was carried out on the basis of an evaluation of the experimental results obtained, which are represented by a combination of chromatogram peaks. The impact of stress factors on microorganisms is determined by the presence of cis-trans-isomerism or cycles of three carbon atoms at the ends of phospholipid fatty acid radicals. In order to verify the objectivity of the results obtained using the proposed method for analysing microorganisms, this work also used the traditional method of controlling soil microorganisms based on the activity of their enzymes – glucosidases, proteases, arylsulphatases and phosphatases. The data obtained indicate the presence of viable soil microorganisms; however, their number does not match the control samples, indicating a failure of the recovery process. The identification results demonstrate the dominance of Gram-negative (Gr⁻) bacteria in the control samples in comparison with the samples in conditions of natural recovery. At the same time, in the process of natural restoration, a greater quantity of fungi was found in the soil. According to the data obtained, the largest amounts of cis – isomers and the value of cyclisation of phospholipid fatty acids are characteristic of microorganisms in the control samples, which may be due to the depletion of the substrate when using the soil for agricultural purposes. The experimental data obtained in the course of this work allow us to propose the procedure as an independent method for conducting biological monitoring of ecosystems when analysing phospholipid fatty acids in the composition of cell membrane lipids.*
Keywords: *soil, microorganisms of soil, fermentative activity, phospholipids*

Анализ микробных фосфолипидов в процессах биомониторинга состояния почв

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Резюме: *Целью данной работы являлась оценка возможности применения метода анализа фосфолипидных жирных кислот в составе липидов клеточных мембран почвенных микроорганизмов для мо-*

ниторинга состояния почв в процессе восстановления. Экспериментальная часть заключалась в определении фосфолипидных жирных кислот (ФЛЖК), включающая экстракцию фосфолипидов органическими растворителями, выделение липидной фракции с помощью полярных силикатных колонок, получение метиловых эфиров жирных кислот путем щелочного метанолиза и анализа содержания ФЛЖК методом газовой хроматографии. Идентификация микроорганизмов осуществлялась на основании оценки полученных экспериментальных результатов, представляющих собой совокупность пиков хроматограммы. Воздействие стрессовых факторов на микроорганизмы определяют по наличию цис-транс-изомерии или циклов из трех атомов углерода на концах радикалов фосфолипидных жирных кислот. С целью проверки объективности результатов, полученных с помощью предлагаемого метода анализа микроорганизмов, в данной работе использовался также традиционный метод контроля почвенных микроорганизмов на основании активности их ферментов – глюкозидаз, протеаз, арилсульфатаз, фосфатаз. Полученные данные свидетельствуют о наличии жизнеспособных почвенных микроорганизмов. Однако их количество не соответствует контрольным пробам, что говорит о незавершении процессов восстановления. Результаты идентификации демонстрируют доминирование грамотрицательных (Гр⁻) бактерий в контрольных пробах в сравнении с пробами в условиях естественного восстановления. При этом в процессе естественного восстановления в почве обнаружено большее количество грибов. Согласно полученным данным, наибольшее количество цис-изомеров и значение циклизации фосфолипидных жирных кислот характерны для микроорганизмов в контрольных пробах, что может быть обусловлено истощением субстрата при использовании почвы в сельскохозяйственных целях. Экспериментальные данные, полученные в ходе данной работы, позволяют предложить использование процедуры анализа фосфолипидных жирных кислот в составе липидов клеточных мембран как самостоятельный метод для проведения биологического мониторинга экосистем.

Ключевые слова: почва, почвенные микроорганизмы, ферментативная активность, фосфолипиды

INTRODUCTION

While the environmental effects of soil pollution manifest themselves later than the results of atmospheric and hydrospheric pollution, the latter can be more persistent and long-lasting[1]. Therefore, the protection of the soil of the biosphere as a whole is possible only on the basis of monitoring the soil environment. Soil microorganisms, which play a key role in soil formation and the maintenance of soil fertility, effecting cycles of all necessary nutrients, also act as decomposers in ecological systems [1].

In order to record the response during biological monitoring at the cellular level, special biomarker molecules – indicators of the state of the organism under study – are used. At the present time, special attention is paid to the use of phospholipid fatty acids in the composition of cell membrane lipids as biomarkers for identifying microorganisms and assessing the influence of stress-factors on them [2].

Phospholipids, which consist of polyols, fatty acid residues and phosphoric acid, are essentially components of living organisms. In the body, phospholipids act as the main component of cell membranes, participating in the processes of nutrition and adaptation. Phospholipids have the following properties, allowing them to be used as biomarkers:

– are not part of the reserve material of the cell;

– form part of the cell membrane, as a result of which they can signal changes in environmental conditions, as well as changes inside the cell;

– high specificity: each group of microorganisms corresponds to a particular fatty acid radical;

– are only contained in the membranes of living organisms; following cell death, phospholipid fatty acids immediately become dephosphorylated[2].

The aim of the research consisted in assessing the possibility of applying the phospholipid fatty acid analysis method in the composition of lipids in the cell membranes of soil microorganisms in order to monitor the state of the soil undergoing processes of recovery from contamination.

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EXPERIMENTAL PART

The object of research in this work consisted of phospholipid fatty acids in the lipid composition of cell membranes of soil microorganisms.

The method for determining phospholipid fatty acids (PFLA) comprises four stages [3, 4]:

1. *Extraction of lipid fraction with organic solvents.* 15 ml of phosphate buffer solution (0,05 mol/l, pH = 7,4), 37,5 ml of methanol and 18,8 ml of chloroform were added to 10 g of sample. Next, the samples were subjected to ultrasonic treatment (15 min) and infused in a rotary mixer (2 h at 10 °C). Following centrifugation (2880 g, 30 min), the supernatant liquid was collected in a separatory funnel. The precipitate was resuspended in 10 ml of the washing mixture (10 ml of chloroform, 8 ml of phosphate buffer, 20 ml of methanol) and centrifuged again (10 min). To the resulting supernatant, which was collected in a separatory funnel, chloroform (18,8 ml) and deionised water (18,8 ml) were added. Following vigorously stirring, the mixture was left to undergo phase separation for 10 hours at ~10 °C.

2. *The isolation of phospholipids from the lipid fraction* was performed using polar silicate columns (Supelclean™ LC-Si, Sigma-Aldrich) under moderate vacuum (elution rate ~1 ml/min). Each column was pretreated with chloroform (3,5 ml) and methanol (3,5 ml) to ensure the purity of the analysis. The sample was concentrated to ~1 ml using a rotary vacuum evaporator and introduced into the column. Undesirable components of the lipid fraction – in particular, triacylglycerides and glycolipids – were separated out using a mixture of chloroform (7 ml) and acetone (28 ml). Polar lipids were eluted with methanol (7 ml), evaporated under nitrogen atmosphere to ~0,5 ml and stored at -30 °C.

3. *Preparation of fatty acid methyl esters* was carried out by means of mild alkaline methanolysis. To the polar lipid solution was added 0,5 ml of a mixture of methanol/toluene (1:1) and, following stirring, 0,5 ml of a freshly-prepared solution of potassium hydroxide (KOH) in methanol (1,122 g of KOH in 10 ml of methanol). The mixture was then heated to 37 °C for 30 min. After cooling, 0,5 ml of acetic acid (0,2 mol/l) was added to the sample. Following shaking, 2 ml of a mixture of hexane: chloroform (4:1) and 2 ml of deionised water were added. Following additional agitation, the mixture was then and centrifuged (2880 g, 10 min). The upper phase was transferred to a clean test tube. The extraction-centrifugation procedure was performed three times. Samples were evaporated in a nitrogen atmosphere to 0,5–1 ml and stored at -30 °C.

4. Qualitative and quantitative analysis of the fatty acid composition of phospholipids was performed by means of gas chromatography using a Varian GC 3800 chromatograph with a VF-5 fused silica capillary (20 m × 0,25 mm × 0,25 µm) and helium as a carrier gas (1 ml/min). The sample was introduced into the evaporator by means of splitless injection (1 µl, 0,2 min, 300 °C). The temperature programme was 50 °C for 1 min; 25 °C/min up to 150 °C, 4 °C/min up to 250 °C, 5 min. A Varian 4000 detector with an ion-cyclotron trap was used (ion transport took place at 280 °C, the collector temperature was 45 °C. The mass limits of the compounds to be determined were in the range of 45–450 amu). The aggregate PFLA quantity was determined as the sum of the peaks in the total ion chromatogram with elution in the retention window from a standard mixture of 26 FAME (Sigma-Aldrich). Quantitative determination was performed using the FAME 11 calibration curves (C_{10}-C_{20}) and the internal standard (methyl nonadecanoic acid, 19:0) [5].

The identification of microorganisms was carried out on the basis of an evaluation of the experimental results obtained, consisting in a combination of chromatogram peaks. To ensure the reliability of identification of PFLA groups, reference samples were used – standard solution 26 FAME (Sigma-Aldrich, USA) with commercial FAME standards manufactured by Sigma-Aldrich and Matreya LLC (USA); nonadecanoic acid methyl ester, 19:0. In the framework of this work, it was necessary to confirm the presence of PFLA radicals, characteristic of certain subgroups of microorganisms (Table 1) [6, 7].

Table 1

Groups of microorganisms and their typical fatty acid radicals [6, 7]

Таблица 1

Группы микроорганизмов и характерные для них радикалы жирных кислот [6, 7]

Group	Subgroup	Fatty acid biomarker
Bacteria	Gr$^+$ Gr$^-$ Other	i14:0, i15:0, a15:0, i17:0, a17:0 cy17:0, cy19:0 16:1ω7t, 16:1ω7, 16:1ω9
Fungi	–	18:2ω6,9

Note. Hereinafter: i – isoacid; a – anteisoacid; cy – cyclic form of the acid; ω – double bond. In the above notation of acids, the first digit indicates the number of carbon atoms, while the second indicates the degree of unsaturation of the radical; the number after ω is the position of the double bond from the methyl end. / Примечание. Здесь и далее: i – изокислота; a – антеизокислота; cy – циклическая форма кислоты; ω – двойная связь. В приведенных обозначениях кислот первая цифра указывает число атомов углерода, вторая – степень ненасыщенности радикала; цифра после ω – положение двойной связи с метильного конца.

The calculation of the values of equivalent chain lengths (ECL) was carried out on the basis of the work of S.A. Mjøs [8], T.K. Miwa et al. [9]. Thus, the presence of anteiso- or isoacids in the final concentrate indicates the presence of Gram-positive (Gr[+]) bacteria, while cyclic acid forms are characteristic of Gram-negative (Gr[-]) bacteria; fungi in the composition of membrane lipids have two double bonds.

The impact of stress factors on microorganisms is determined by the presence of cis-trans-isomerism or cycles of three carbon atoms at the ends of phospholipid fatty acid radicals.

In order to verify the objectivity of the results obtained using the proposed method for analysing microorganisms, the present work also used the traditional method for monitoring soil microorganisms based on the activity of their enzymes – glucosidases, proteases, arylsulfatases, phosphatases. [10, 11]. The analysed enzymes are of interest, since they determine the circulation of the most essential nutrients for plants, such as carbon, nitrogen, sulphur and phosphorus.

The subject of the research comprised soil samples selected from the Radovesice district near the city of Usti nad Labem (Czech Republic). This area had previously been used for coal mining and was then backfilled with soil.

Soil sampling was carried out in 4 replications from an average depth of 5 cm (since it is at this depth to which the roots of most plants extend and where the majority of soil microorganisms actively develop) [12].

In order to establish the possibility of applying the analysis of phospholipid fatty acids to assess the state of soil microorganisms, samples were taken from various locations:

– undergoing reclamation process including artificial tree planting;

– undergoing natural recovery, associated with the process of succession under the influence of natural factors only and in the absence of any particular interventions.

Soil from agricultural land acted as a control (following the completion of restoration processes, it was planned to use the soil in agriculture).

In order to ensure statistically reliable results, multiple sampling was carried out from each site of the surveyed territory. Statistical processing was performed using standard procedures of the Microsoft Excel application package. In this way, variance analysis methods were used together with determinations of the average value, standard deviation, confidence interval and relative error.

RESULTS AND DISCUSSION

As a result of the studies carried out using the above methods, phospholipid fatty acids and related substances were identified in the samples (Table 2).

Table 3 shows the quantitative (in mg) content of fatty acids, which are biomarkers in terms of absolutely dry matter (ADM) of the studied samples, as well as the limits of errors in their determination.

Table 2

**Identified PLFA in the studied samples with retention time
and equivalent chain length (ECL)**

Таблица 2

**Идентифицированные ФЛЖК в исследованных пробах с указанием времени
удерживания и эквивалентных длин цепей (ECL)**

PLFA	Time holding, min	ECL	PLFA	Time holding, min	ECL
10:0	5,716	9,9263	16:0	15,543	16,0014
12:0	7,281	11,9963	**i-17:0**	16,891	16,6837
12:1	7,681	11,8350	**a-17:0**	17,086	16,7904
13:0	9,529	13,0043	**cy17:0**	17,321	16,9024
13:1	9,546	12,8460	17:0	17,607	17,0324
14:0	11,521	13,9946	2-OH 16:0	18,233	17,3134
i-15:0	12,261	14,6309	**18:2ω6,9**	19,089	17,7390
a-15:0	12,881	14,1706	18:1ω9	19,215	17,7624
15:1	13,045	14,7786	**18:1ω7**	19,342	17,8661
15:0	13,486	14,9979	18:0	19,793	18,0021
2-OH 14:0	14,248	15,1719	10-Me 18:0	20,605	18,5201
3-OH 14:0	14,753	15,6420	19:1	21,313	18,8269
i-16:0	15,086	15,7950	**cy19:0**	21,566	18,8304
16:1ω7t	15,149	15,8315	19:0	21,887	19,0041
16:1ω9	15,233	15,8587	20:0	23,363	20,0073
16:1ω7	15,311	15,8622	–	–	–

Biomarkers are in bold, PLFA 10:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0 are standards (see «Objects and methods of research») / Биомаркеры выделены жирным шрифтом, ФЛЖК состава 10:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0 являются стандартами (см. «Объекты и методы исследований»)

In accordance with the data given in Table 1, the content of PLFA in the composition of the cell membranes of microorganisms per ADM of the studied samples was determined (Table 4).

According to the results of PLFA determination, it should be noted that the number of microorganisms in the biocenosis of the studied soils differs from their number in the control sample, indicating the incompleteness of the processes of soil restoration (Table 5, Fig. 1).

Table 3

Quantitative content and error limits in the determination of fatty acids acting as markers

Таблица 3

Количественное содержание и пределы погрешностей при определении жирных кислот, выступающих в роли маркеров

Marker	Quantity of PLFA, mg/g ADM		
	Control	Reclamation	Natural recovery
i-15:0	3,01 ± 0,15	2,03 ± 0,09	1,12 ± 0,02
a-15:0	2,58 ± 0,11	0,96 ± 0,01	0,61 ± 0,01
16:1ω7t	not open	not open	not open
16:1ω9	0,97 ± 0,05	not open	not open
16:1ω7	1,83 ± 0,08	1,62 ± 0,01	0,73 ±0,03
i-17:0	0,58 ± 0,03	0,38 ± 0,01	0,21 ± 0,01
a-17:0	0,35 ± 0,01	0,26 ± 0,01	0,19 ± 0,01
cy17:0	2,71 ± 0,01	1,28 ± 0,01	0,44 ± 0,01
18:2ω6,9	5,22 ± 0,52	1,17 ± 0,26	0,42 ± 0,16
cy19:0	4,69 ± 0,22	2,71 ± 0,001	1,51 ± 0,002

Table 4

Phospholipid fatty acid (PLFA) content in the studied samples

Таблица 4

Содержание фосфолипидных жирных кислот (ФЛЖК) в исследованных образцах

Sample	Quantity of PLFA, mg/g ADM				
	General	Bacteria		Fungi	Other
		Gr$^+$	Gr$^-$		
Control	22,02 ± 5,73	6,02 ± 2,03	7,96 ± 3,52	5,23 ± 0,52	2,81 ± 0,81
Reclamation	12,05 ± 2,46	3,75 ± 0,83	3,99 ± 1,08	1,19 ± 0,26	1,64 ± 0,38
Natural regeneration	6,01 ± 2,39	2,19 ± 0,64	1,97 ± 0,71	0,41 ± 0,16	0,78 ± 0,32

Table 5

Number of isomerised phospholipid radicals

Таблица 5

Количество изомеризованных фосфолипидных радикалов

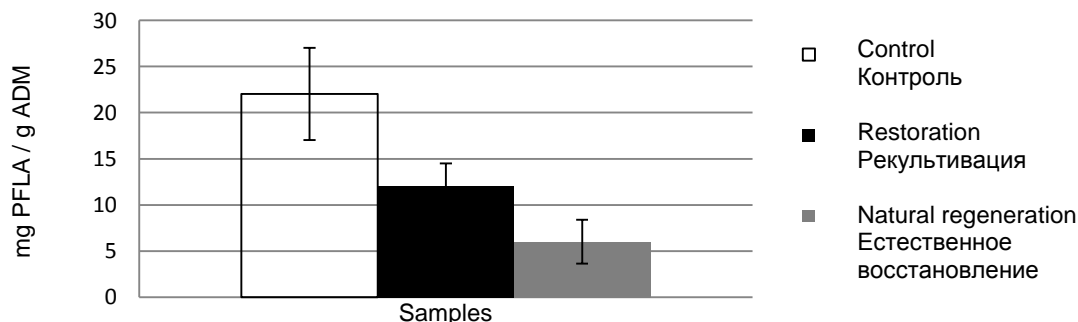| Sample	Number of isomers, mg/g ADM			
	Cis-isomers	Trans-isomers	Cyclic forms	Precursors of cyclic forms
Control	6,94 ± 0,35	not open	2,78 ± 0,34	6,93 ± 0,54
Reclamation	2,37 ± 0,22	not open	0.57 ± 0,03	2,38 ± 0,12
Natural regeneration	1,38 ± 0,07	not open	0,26 ± 0,08	1,37 ± 0,17

Fig. 1. Total phospholipid fatty acid content in soil samples

Рис. 1. Общее содержание фосфолипидных жирных кислот в почвенных образцах

The estimation of the microorganism content according to the results of the identification of characteristic groups of PLFAs (Fig. 2, see Table 2) demonstrates the dominance of Gr⁻ bacteria in the control sample in comparison with samples under conditions of natural recovery. At the same time, during the process of natural restoration, a greater quantity of fungi was found in the soil. Minor discrepancies in the results of the control and recultivated systems are due to the fact that the recultivation involves the rapid restoration of the soil to a condition suitable for use in agriculture.

However, it should be noted that the process of natural restoration implies succession, i.e. a consistent replacement of some biological communities by others. The obtained results confirm the longer duration of the natural restoration of soil fertility.

The influence of stress factors on soil microorganisms leads to the formation of cis-trans-isomers or cycles of three carbon atoms at the ends of fatty acid radicals (see Table 5). For bacteria that already have cyclic fatty acid radicals in cellular membranes (Gr- i.e. cycles of 17 and 19 carbon atoms), the number of cyclic forms precursors (radicals with double bonds of 16 and 18 carbon atoms) is analysed.

According to the data obtained, the largest quantity of cis-isomers and the highest cyclisation value of FLFA are noted for microorganisms in the control system, which may be due to the effect of a combination of adverse factors in agricultural production (use of chemical plant protection products, fertilisers, etc.). The smallest amount of cis-isomers is characterised by the soil in the process of natural restoration, since this process involves a flow of successive processes under natural conditions.

Table 6 shows the activity values of four groups of microbial enzymes for the studied soil samples, confirming the data on the PLFA content in the composition of their cell membranes.

It is noted that the activity of the studied microbial enzymes under the conditions of the natural restoration of the soil is lower than in the samples of the control and recultivated soil. In this case, the values of the enzymatic activity of microorganisms in the reclaimed soils are close to those of control samples. An exception to this is seen in the proteolytic activity of microorganisms (see Table 6).

Higher values of protease activity in soil samples taken from the recultivated areas compared to the control sample may be due to the uneven distribution of microorganisms in the soil.
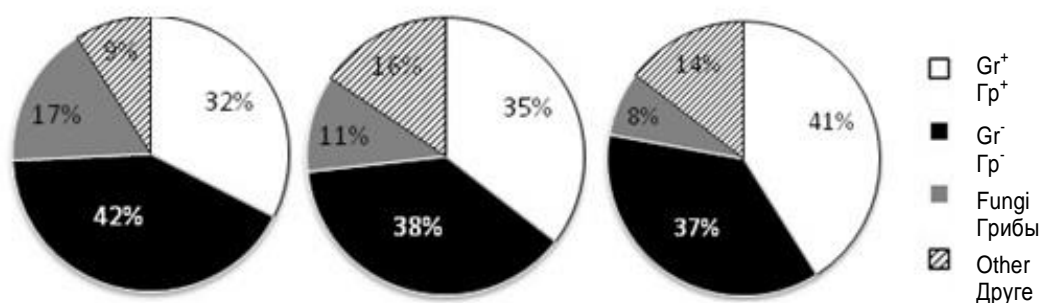

***Fig. 2. Ratio of microbial groups in soil samples, from left to right:
control sample, samples from the restoration areas
and from the areas of natural regeneration***

***Рис. 2. Соотношение микробных групп в почвенных образцах, слева направо:
контрольная проба, пробы с территорий рекультивации
и с территорий естественного восстановления***

Table 6

Enzymatic activity values of soil microorganisms

Таблица 6

**Значения ферментативной активности
почвенных микроорганизмов**

Enzyme	Activity value, nmol/min /g ADM		
	Control	Reclamation	Natural recovery
Glucosidase	0,0057 ± 0,0024	0,0050 ± 0,0017	0,0035 ± 0,0010
Protease	0,0205 ± 0,0071	0,0362 ± 0,0029	0,0125 ± 0,0040
Arylsulfatase	0,0159 ± 0,0066	0,0040 ± 0,0007	0,0021 ± 0,0008
Phosphatase	0,1317 ± 0,0347	0,0742 ± 0,0280	0,0313 ± 0,0180

CONCLUSION

In the course of the study, results of the analysis of phospholipid fatty acids in the lipid composition of cell membranes of microorganisms of soil ecosystems, which correlate with indicators of the enzymatic activity of microorganisms, were obtained.

The total PLFA and enzymatic activity values obtained indicate the presence of viable cells with a full cell wall and a set of active enzymes in all soil samples in the microbial communities; a significant stress effect on the microbial ecosystems in the case of long-term production processes in a given territory is also indicated. It was empirically demonstrated that the minimum amount of phospholipid fatty acids in the composition of lipids of cell membranes is typical for soil samples under processes of natural restoration and recultivation, i.e. 6 and 3 times smaller compared to control samples respectively. A similar dependence is noted for the activity values of the studied enzyme groups. Based on the data obtained, it is concluded that the recovery processes are not complete.

In addition, the high content of isomerised phospholipid radicals (cis-isomers and cyclic forms) for control soil samples indicates a significant stress effect on microbial communities in the development of natural areas for agricultural production.

Despite the labour-intensive nature of the analysis of phospholipids, this complex method allows detailed information to be obtained about the systems under study, including: determining the number of living microorganisms, identifying groups of microorganisms in the microbial communities and evaluating the influence of external factors on the microbiocenosis. The experimental data obtained in the course of this work allow us to propose the use of analysis procedures of phospholipid fatty acids forming part of cell membrane lipids for the development of theory and to support environmental monitoring in order to preserve and restore soil fertility on agricultural lands and as part of rural landscapes.

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Contribution

Aida R. Khabibullina, Tatyana V. Vdovina, Alexander S. Sirotkin, Josef Trögl, Taťjána Brovdyová, Pavel Kuráň carried out the experimental work, on the basis of the results summarized the material and wrote the manuscript. Aida R. Khabibullina, Tatyana V. Vdovina, Alexander S. Sirotkin, Josef Trögl, Taťjána Brovdyová, Pavel Kuráň have equal author's rights and bear equal responsibility for plagiarism.

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Хабибуллина А.Р., Вдовина Т.В., Сироткин А.С., Трёгл Й., Бровдыова Т., Куран П. выполнили экспериментальную работу, на основании полученных результатов провели обобщение и написали рукопись. Хабибуллина А.Р., Вдовина Т.В., Сироткин А.С., Трёгл Й., Бровдыова Т., Куран П. имеют на статью равные авторские права и несут равную ответственность за плагиат.

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