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Comparative assessment of two calculation methods for change dynamics in the major fatty acid content of *Triticum aestivum* L. wheat calli under the action of low-intensity laser radiation

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Abstract: *The aim of the present work involves a comparative analysis of the changes in the absolute content of the main fatty acid (FA) component of the total lipid amount in wheat calli (Triticum aestivum L.) under the action of low-intensity laser radiation (LILR), as well as an assessment of the influence of the FA proportion calculation method on the resultant picture of the FA change dynamics. The content of major FAs in callus tissue and its changes when exposed to LILR were determined using gas chromatography-mass spectrometry. A comparative analysis of the calculation results of the absolute (μg/g dry weight) and relative (in %wt. of the sum of the areas of chromatographic peaks) content of the target components (palmitic, stearic, linoleic and linolenic acids) was carried out. When comparing the two calculation methods, no qualitative differences in the change dynamics were observed. At the same time, significant differences were found between the quantitative indicators of the FA content in absolute and relative units. For example, the absolute amount of monounsaturated oleic acid five minutes increased by 23% following the removal of laser radiation, while the relative amount increased by only 14%. For polyunsaturated linoleic and linolenic acids, this difference was even more significant. In general, the trends in changes caused by irradiation in the main fatty acid content compared with the total callus lipids appear to be similar to the stress response of plant tissue to adverse environmental conditions. The scope of two methods for calculating the content of FAs is also discussed.*

Keywords*: laser stimulation, callus, fatty acids, Triticum aestivum* L.*, quantitative analysis*

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Сравнительная оценка двух способов расчета динамики изменений содержания главных жирных кислот в каллусах пшеницы (*Triticum aestivum* L.) при действии низкоинтенсивного лазерного излучения

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Резюме: *Целью представляемой работы являлся сравнительный анализ изменений абсолютного содержания главных жирных кислот (ЖК) суммарных липидов каллусов пшеницы (Triticum aestivum L.) при действии низкоинтенсивного лазерного излучения (НИЛ), а также оценка влияния способа расчета количества ЖК на конечную картину динамики изменений этого показателя. Методом*

газовой хромато-масс-спектрометрии определено содержание главных ЖК в каллусной ткани и его изменения при облучении НИЛ. Проведен сравнительный анализ результатов расчета абсолютного (мкг/г сухого веса) и относительного (в % вес. от суммы площадей хроматографических пиков) содержания целевых компонентов: пальмитиновой, стеариновой, линолевой и линоленовой кислот. Установлено, что характер динамики изменения содержания этих соединений при облучении, оцененный двумя способами, не имеет качественных различий между вариантами расчета. Направленность изменений в содержании главных жирных кислот суммарных липидов каллусов, вызванных облучением, в целом имеет сходство со стрессовой реакцией растительной ткани на неблагоприятные условия внешней среды. При этом обнаружены существенные различия между количественными показателями содержания ЖК в абсолютных и относительных единицах. Например, увеличение абсолютного количества мононенасыщенной олеиновой кислоты через пять минут после снятия воздействия лазерного излучения составило 23%, в то время как относительное количество увеличилось за то же самое время только на 14%. Для полиненасыщенных линолевой и линоленовой кислот эта разница была еще более значительной. Обсуждается область применения двух способов расчета содержания ЖК.

***Ключевые слова**: лазерная стимуляция, каллус, жирные кислоты, Triticum aestivum L., количественный анализ*

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INTRODUCTION

The phenomenon of low-intensity laser radiation in the infrared and visible ranges having a stimulating effect not only on animal tissue and microorganisms [1], but also on plants, including seeds and plant tissue culture, can be considered as demonstrated in fact [1, 2]. While the effects of this type of radiation mechanism have been studied in detail in relation to animal and human organisms, more research is required regarding their effect on plant tissues. When presenting the recorded results of the stimulating laser effect, the information presented in the literature often consists of phenolmenological description, while possible approaches to realising this effect on plant tissues remain poorly studied [3–5]. At the present time, there are no reliable data on the key compounds that act as indicators when analysing the response of the plant to the action of this type of radiation. The existence of plants, their reproduction, growth, development and adaptation is well known to be heavily influenced by light, namely the frequency and intensity of illumination and the spectral characteristics of the radiation. Therefore, the study of the effect of low-intensity laser radiation on plants is of interest not only for optimising its use in a practical setting, but also for studying the laws of the action of light on plant organisms as a whole. Due to the physical properties of this radiation, well-controlled conditions can be applied in such studies. The body's reaction to the effects of various stressors (in this case, excessive light intensity) is well known to primarily cause changes in membrane structures, including the composition and content of lipids and fatty acids. As is known, fatty acids are among the most dynamic components of lipid molecules, actively changing their composition and amount in response to stressors. In this case, not only may shifts occur in the ratio of various groups of fatty acids, but their degree of unsaturation[1] may also change. These indicators vary widely with temperature, illumination intensity and the concentration of osmotic substances and salts. We considered the changes in the fatty acid composition of wheat callus lipids under the influence of laser light to be further evidence of the low-intensity laser radiation effect on plants causing a reaction similar to stress. Earlier, data were obtained by us (using gas chromatography-mass spectrometry) on the relative content of fatty acids in wheat callus lipids. At the initial stage of tissue reaction (immediately after removal of the laser), a decrease was noted in the degree of unsaturation of fatty acids, mainly due to a decrease in the content of polyunsaturated acids [6]. Such an apparently non-specific reaction is similar to changes in the structure and chemical composition of lipids in response to various stressful stimuli. The fatty acid content in this study was determined as a percentage of the sum of the chromatographic peak areas taking into account the response of each acid. This method has long been applied to quantify the content of fatty acids when studying lipid metabolism. However, in

[1]Чиркова Т.В. Физиологические основы устойчивости растений: учеб. пособие для студентов биологических факультетов вузов. СПб: Изд-во СПбГУ, 2002. 244 с.

making such an assessment, a decrease in the content of one acid inevitably leads to an increase in the content of other acids, regardless of the actual absolute changes in their quantity. Subsequently, for this reason, researchers began to appeal to traditional quantitative methods of FA analysis, such as gravimetry and the absolute calibration method, simultaneously taking into account the response of the internal standard in the case of gas chromatographic analysis.

Due to possible errors in the FA relative determination, it was logical to verify the results we obtained earlier by analysing the absolute content of these acids in similar samples under the same experimental conditions and determine the applicability limits of the semiquantitative (relative) and quantitative (absolute) methods of gas chromato-graphic analysis of the fatty acid content. Therefore, the aim of the present work lies in the comparative analysis of changes in the absolute content of the main FA total lipids in wheat calli under the action of low-intensity laser radiation.

EXPERIMENTAL PART

The focus of the study is skala breed wheat calli. Mature embryos with half of the endosperm were used as an explant; callusogenesis was induced on a modified MS medium (Murashige, Skoog) with the addition of 2% sucrose and 2 mg of 2,4-D. Calli for irradiation and subsequent analysis of lipids and fatty acids were sampled on the 2nd day after transplantation for 1 passage. Irradiation was carried out according to the method developed at the Siberian Institute of Plant Physiology and Biochemistry, using a helium-neon laser with the following parameters: 632.8 nm radiation wavelength, 5 min irradiation time, 3.6 J/cm^2 irradiation dose. This dose was previously identified as able to stimulate callusogenesis and morphogenetic processes in plant tissue. For lipid extraction, plant material of a known weight (0.2 g) was fixed in liquid nitrogen, then 0.001% ionol was added and stirred to obtain a homogeneous mass [7]. Subsequently, 10 ml of chloroform and methanol mixture was added in a volume ratio of 1:2, mixed thoroughly and left for 30 minutes until the lipids had completely diffused into the solvent. The solution was transferred quantitatively by filtration to a separator funnel with triple washing of the mortar and filter using the same solvent mixture. For better delamination, water was added. The extraction was carried out three times. For analysis of total lipids, the lower chloroform fraction was separated. Chloroform (ultra-high purity, stabilised by 0.005% amylene) from the lipid extract was removed under vacuum using an RVO-64 rotary evaporator (Czech Republic). FA methyl esters (FAME) were obtained according to the method presented in [8]. An additional purification of FAME was performed by TLC in a chamber with benzene on glass plates with KSK silica gel (Russia) in mobile phase (R_f = 0.71–0.73). In order to visualise the FAME zone, the plates were sprayed with 10% H_2SO_4 in MeOH and heated in an oven at 100 °C. The FAME zone was removed from the plate with a spatula and eluted from silica gel by (*n*)-hexane. FAME analysis was performed by GLC using a 5973/6890N MSD/DS chromatography mass spectrometer (Agilent Technologies, USA) with the quadrupole mass spectrometer as a detector, electron impact in terms of the ionisation method with the ionisation energy of 70 eV. The analysis was carried out in the recording mode of the total ion current. In order to separate the FAME mixture, an HP-INNOWAX capillary column (30 m × 250 μm × 0.50 μm) with a stationary phase (PEG) was used. The carrier gas was helium; the gas flow rate was 1 ml/min. Temperature, °C: evaporator – 250; ion source – 230; detector – 150; the line connecting the chromatograph to the mass spectrometer – 280. The scanning range is 41–450 a.e.m, the volume of the injected sample is 1 μl, the splitter is 5:1. Separation of the FAME mixture was carried out in isothermal mode at 200 °C. Mass spectra of standard compounds were used to identify FAs using MSD ChemStation D.02.00.275 software (Agilent Technologies).

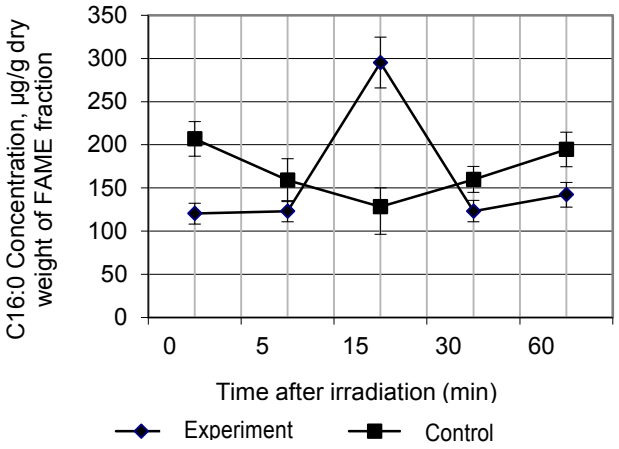
The relative content of FA was determined using the method of internal normalisation as a percentage (% wt.) of their total content in the sample, taking into account the FA response coefficient. The absolute content of the FAME fraction was determined by weighing using a GR-120 electronic balance (A&N Company Ltd., Japan), the sample was dried to constant weight.

Quantitative analysis of the main FAs was carried out using the absolute (external calibration) method; nonadecanoic acid (C19:0), not contained in our samples, was used as the internal standard by its addition in a known amount at the homogenisation stage. An existing fatty acid standard (Supelco® 37 Component FAME Mix, USA) was used for calibration.

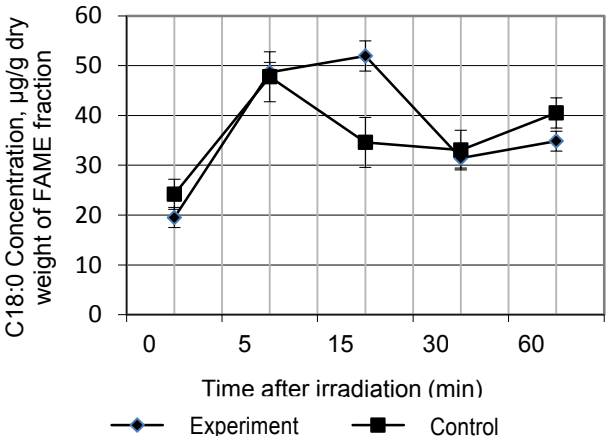
The content of FA was determined for 100 calli in the experiment and in the control in three independent experiments. The paper provides mean values and their standard deviations. The signifycance of differences in the compared mean values was evaluated using the *t*-criterion ($P < 0.05$). The distribution hypothesis was checked using the Shapiro-Wilk test.

RESULTS AND DISCUSSION

The results of the analysis of the change dynamics in the content of the main FAs of *T. aestivum* total callus lipids, such as palmitic (C16:0), stearic (C18:0), oleic (C18:1ω9), linoleic (C18:2ω6) and linolenic (C18ω:3) acids, are presented in the figure.



a



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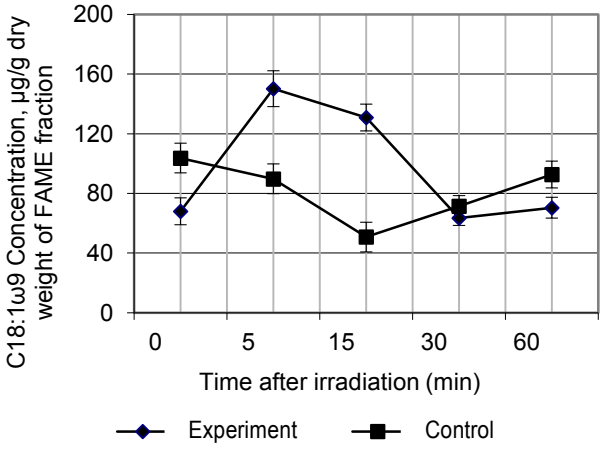

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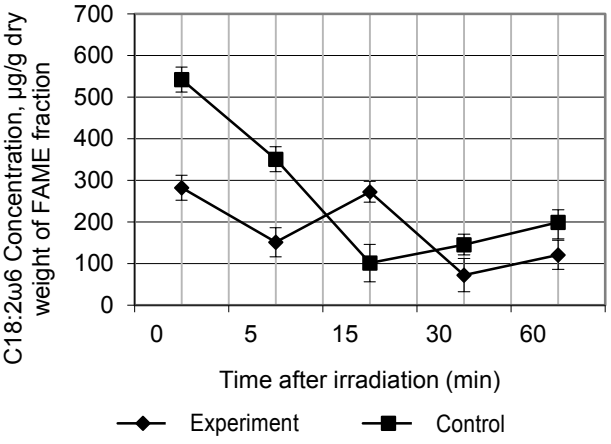

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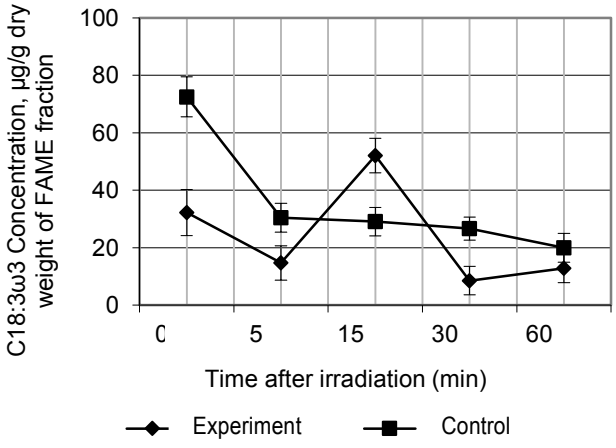

e

Change dynamics in the absolute content of fatty acids total callus lipids within one hour after irradiation with low-intensity laser light (He-Ne, λ = 632.8 nm): a – palmitic acid (C16: 0); b – stearic acid (C18: 0); c – oleic acid (C18: 1ω9); d – linoleic acid (C18: 2ω6); e – linolenic acid (C18: 3ω3)

Динамика изменений абсолютного содержания ЖК суммарных липидов каллусов в течение часа после облучения низкоинтенсивным светом лазера (He-Ne, λ=632,8 нм): a – пальмитиновая кислота (C16:0); b – стеариновая кислота (C18:0); c – олеиновая кислота (C18:1ω9); d – линолевая кислота (C18:2ω6); e – линоленовая кислота (C18:3ω3)

As can be seen from the figure, for palmitic acid, the most significant differences between the experiment and the non-irradiated control in its content were observed 15 minutes following the removal of the radiation source (Fig. *a*). At this moment, its noticeably higher content in irradiated calli was recorded in comparison with the control. With the exception of this point, the palmitic acid content in the experiment was lower than in the control during the observation period. Another main saturated acid, stearic acid (Fig. *b*), also demonstrates a maximum content (more than 1.5 times higher than the control) in the irradiated samples at the 15th minute of observation. As for polyunsaturated acids, the most significant differences between the experiment and the non-irradiated control were observed in the content of monounsaturated oleic acid. Stearoyl CoA desaturase (Δ9 desaturase) is known to play a central role in lipid metabolism [9]. It induces the formation of the first *cis*-double bond between the 9 and 10 carbon atoms in palmitic and stearic acids, which are converted to palmitoleic (C16:1Δ9) and oleic acid (C18: Δ9), respectively. In addition to the fact that these FAs function as components of lipids, they also serve as intermediaries in signalling, including the formation of stress reactions and in the processes of cell differentiation [10, 11]. Therefore, the activity of this desaturase was of particular interest to us in studying the effect of laser light on the fatty acid composition of wheat callus tissue lipids. As can be seen from the figure, immediately after irradiation, the oleic acid content was significantly lower than in the control, then at the 5th and especially at the 15th minute, the oleic acid content was measured as being significantly higher (2–2.5 times higher) in the irradiated calli. A decrease in content at an early stage (immediately following exposure) of the response to radiation is characteristic of all the analysed acids and is similar to the stress response of plants to various adverse environmental conditions [12]. The subsequent (5th minute post-exposure) increase in the oleic acid content indicates an increase in the activity of Δ9-desaturase, which is also characteristic of the response of plant tissue to the action of stressors [13]. It should be noted that the change dynamics in the oleic acid content differed from that of other major unsaturated acids. An increase in its content started earlier and was more pronounced than for diene linoleic and triene linolenic acids. In agreement with the earlier analysis of the relative content of FAs, the oscillatory nature of changes in the content of major fatty acids in callus tissues under the action of low-intensity laser radiation was noted in the present work. Moreover, for irradiated calli, the amplitude of such oscillations was generally larger than in unirradiated controls. Such an increase is also characteristic of the primary stress response. A similar reaction to irradiation was demonstrated by studying the dynamics of lipid peroxidation under the action of laser light [14].

The table presents a comparative analysis of the results of two methods for calculating the content of fatty acids in the tissues of wheat calli 5 minutes following exposure.

The presented values characterise the percentage of the sum of the areas of the chromatographic peaks of the FA and of their total absolute content in the first and the second case, respectively. The sum of minor acids is given for comparison with the content of major components. In terms of qualitative differences between the irradiated and control samples, the nature of the change dynamics (i.e. an increase or decrease in the fatty acid content) is generally consistent with both methods for calculating the FA content in the total lipids of the studied plant tissues. However, noticeable quantitative differences were found. From the data presented in the table, it can be seen that the percentage of saturated FAs in callus lipids calculated from the sum of the areas of chromatographic peaks is equal to or slightly higher than the values calculated in accordance with the absolute amount of these acids.

Relative and absolute content of the main fatty acids of total lipids in calli of Triticum aestivum L. wheat

Относительное и абсолютное содержание главных жирных кислот суммарных липидов в каллусах пшеницы Triticum aestivum L.

Acid	Experiment* (% wt.)	Control** (% wt.)	Experiment* (% μg/g dry weight)	Control** (% μg/g dry weight)
C16:0(palmitic)	26.4±3.1	23.8±2.7	37.1±2.4	9.2±2.1
C18:0(stearic)	7.0±0.5	4.8±0.6	9.5±0.4	6.8±0.3
C18:1(n-9) (oleic)	7.3±0.6	6.3±0.5	19.1±0.3	14.7±1.1
C18:2(n-6) (linoleic)	38.1±2.9	47.1±3.9	21.8±1.6	29.9±1.9
C18:3(n-3) (α-linolenic)	6.1±0.8	10.1±1.1	2.6±0.3	5.5±0.4
Sum of minor acids	15.1±1.2	7.9±1.4	9.9±0.9	10.2±1.1

*The relative and absolute contents were calculated in the irradiated and control samples 5 minutes after removal of the low-intensity laser radiation: * – as a percentage of the sum of the areas of chromatographic peaks, ** – as a percentage of the total absolute weight of the FAME fraction taken as 100%. Data on the relative content of FAs were obtained by the authors of this article earlier and published in [6].*

At the same time, for mono- and polyunsaturated acids, there were significant discrepancies in the results of a quantitative assessment of their content between the two methods of analysis. Thus, an increase in the oleic acid content 5 minutes after irradiation comprised 23%, while the relative amount increased only by 14% (the difference was 9%). For linoleic and linolenic acids, the difference in content between the two methods of analysis was equal to 8 and 13.2%, respectively. In other words, the method of relative calculation of the FA provides only a preliminary assessment of trends in their composition and content, while data on the absolute content naturally more accurately and correctly reveal the features of these changes and allow for the assessment of the real biochemical shifts caused by irradiation.

CONCLUSION

An analysis of the change dynamics in the content of major fatty acids of the total lipids in wheat calli under the action of low-intensity laser radiation, evaluated using two methods, show an absence of any significant qualitative differences. In both methods of FA content analysis, the change dynamics were similar to the stress response of the plant. However, the analysis of shifts in the absolute content of fatty acids during irradiation provides a more accurate quantitative assessment of changes in the composition and content of these components under the action of laser light, thereby providing important information on changes in the lipid metabolism of plant tissue caused by the irradiation effect. Nevertheless, from our point of view, the relative method can be used for preliminary analysis and development of experimental research strategies.

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Contribution

Lyubov V. Dudareva, Vladimir N. Shmakov, Elena G Rudikovskaya carried out the experimental work, on the basis of the results summarized the material and wrote the manuscript. Lyubov V. Dudareva, Vladimir N. Shmakov, Elena G. Rudikovskaya have equal author's rights and bear equal responsibility for plagiarism.

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