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Regulation of the activity of adenylate cyclases by hydrogen peroxide in pea root cells Infected with pathogens and a mutualist

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Abstract: *This study examines the effect of a range of exogenous concentrations of hydrogen peroxide on the activity of transmembrane and soluble adenylate cyclases (EC 4.6.1.1) contained in root cells of pea seedlings infected with one of the following: Rhizobium leguminosarum bv. Viciae, Pseudomonas syringae pv. Pisi, and Clavibacter michiganensis ssp. sepedonicus. The results showed that the pool of intracellular H_2O_2 increased when pea roots were infected with bacteria regardless of type. The study analysed the concentration of intracellular cyclic adenosine monophosphate, a product of the adenosine triphosphate cyclization reaction catalyzed by transmembrane and soluble adenylate cyclases. The concentration of intracellular cyclic adenosine monophosphate increased when infected with either Rhizobium leguminosarum bv. viciae or Clavibacter michiganensis ssp. Sepedonicus; however, the concentration decreased by 20% when infected with Pseudomonas syringae pv. Pisi. The in vitro activity of soluble and transmembrane adenylate cyclases from pea root cells inoculated with Rhizobium leguminosarum bv. viciae was H_2O_2 dose-dependent: 100 nM of H_2O_2 reduced the activity of soluble and transmembrane adenylate cyclases slightly, while 26 μM inhibited their activity by 50–60%. When infected with Pseudomonas syringae pv. pisi, the reduction in the activity of soluble and transmembrane adenylate cyclases was independent of the concentrations of H_2O_2 in the range investigated. When infected with Clavibacter michiganensis ssp. sepedonicus, 100 nM of H_2O_2 inhibited the activity of transmembrane adenylate cyclases, although enhancing the activity of soluble adenylate cyclases. On the contrary, concentrations of H_2O_2 of 2.6 and 26 μM increased the activity of transmembrane adenylate cyclases and inhibited the activity of soluble adenylate cyclases. It can be concluded that the specific concentration of second messengers in plant cells depends on the specificity of the biotic stressor and forms, inter alia, by their mutual influence on the components of other plant signaling systems.*

Keywords: *hydrogen peroxide, soluble adenylate cyclase, transmembrane adenylate cyclase, mutualist, pathogens*

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УДК 581.1

Регуляция активности аденилатциклаз клеток корня гороха пероксидом водорода при инфицировании патогенами и мутуалистом

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Резюме: *Целью данной работы являлось изучение влияния различных экзогенных концентраций пероксида водорода на активность трансмембранной и растворимой аденилатциклаз (КФ 4.6.1.1) в клетках корня проростков гороха, инфицированного Rhizobium leguminosarum bv. viciae, Pseudomonas syringae pv. Pisi или Clavibacter michiganensis ssp. sepedonicus. Исследования показали, что пул внутриклеточного H_2O_2 повышался при инфицировании корней гороха всеми ука-*

занными бактериями. Концентрация внутриклеточного циклического аденозинмонофосфата, продукта реакции циклизации аденозинтрифосфата, катализируемой трансмембранной и растворимой аденилатциклазами, в тех же образцах при инфицировании Rhizobium leguminosarum bv. *Vicea или Clavibacter michiganensis* ssp. *sepedonicus также возрастала; а под воздействием Pseudomonas syringae* pv. *pisi снижалась на 20%. Активность трансмембранной и растворимой аденилатциклаз in vitro из инокулированных Rhizobium leguminosarum* bv. *vicea клеток корня гороха при добавлении различных концентраций H_2O_2 изменялась дозозависимо: 100 нМ H_2O_2 незначительно снижали их активность, в то время как 26 мкМ ингибировали активность на 50–60%. На фоне инфицирования Pseudomonas syringae* pv. *pisi добавление любой из выбранных концентраций H_2O_2 в равной степени снижало активность трансмембранной и растворимой аденилатциклаз. При инфицировании Clavibacter michiganensis* ssp. *sepedonicus 100 нМ H_2O_2 ингибировали активность трансмембранной аденилатцтклазы, но оказывали активирующий эффект на растворимую аденилатциклазу. Напротив, концентрации H_2O_2 2,6 и 26 мкМ повышали активность трансмембранной аденилатцтклазы и ингибировали активность растворимой аденилатциклазы. Сделан вывод о том, что определенный концентрационный статус вторичных мессенджеров в клетках растений зависит от специфичности биотического стрессора и формируется, в том числе, путем их взаимного влияния на компоненты других сигнальных систем растений.*

Ключевые слова: *пероксид водорода, растворимая аденилатциклаза, трансмембранная аденилатциклаза, мутуалист, патогены*

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INTRODUCTION

Second messengers of signaling systems participate in the regulation of plant metabolism at all stages of development and are subject to the influence of external agents, for example in biotic stress. The adenylate cyclase signaling system is actively involved in plant stress and defense responses [1, 2]. Earlier, we showed that the degree of activity of cyclic adenosine monophosphate (cAMP) and adenylate cyclase in root cells changed significantly 5 minutes after infection of the root of pea seedling with various agents [1, 3], viz. different strains of *Rhizobium leguminosarum bv. viciae (Rlv)* and phytopathogenic bacteria which differ by specialization, *Pseudomonas syringae bv. pisi* (*Psp*), the pathogen of peas, and *Clavibacter michiganensis sps. sepedonicus (Cms)*, a specific pathogen for potato. Moreover, the strength of activity of transmembrane adenylate cyclases (TACs) and soluble adenylate cyclases (SACs) was dependent on the infectious agent, despite the non-specific interaction; infection with *Rlv* led to the most pronounced activation of TACs and SACs in pea root cells compared with the activation following infection with *Psp* and short-term contact with *Cms* [1, 3]. Possible mechanisms for modulating the activity of both forms of adenylate cyclases (ACs) under biotic stress may include both ligand-receptor interactions [4] and the influence of calcium ions [5]. The latter can act as an intracellular second messenger, and its concentration can change rapidly following infection. It should be noted that, in the early stages of biotic stress, the concentration of H_2O_2, another signal molecule, has already rapidly and sharply increased in the apoplast and intracellular space of plant cells [6–8], which is likely to cause analagous changes in the activity of both forms of ACs. However, knowledge of the effect of H_2O_2 molecules on the activity of transmembrane and soluble forms of AC in plant cells is isolated to specific examples and therefore incomplete: for example, it was shown that 0.2–0.6 μM of H_2O_2 had only a minor effect on TACs and SACs from the vacuoles of beetroot parenchyma cells during various periods of root crop dormancy under long-term biotic stress [9]. Interpretation of these results was complicated by the physiological peculiarities of the beetroot crop (a biennial plant with associated dormant periods), hence it was considered more efficacious to continue the study on the root of pea seedlings. Therefore, the purpose of this study was to investigate the effect of the second messenger in the superoxide synthase signaling system (H_2O_2) on the activity of transmembrane and soluble forms of AC from pea root cells following inoculation with *Rlv, Psp*, and *Cms.*

EXPERIMENTAL PROCEDURE

The subjects were 3-day-old pea seedlings (*Pisum sativum*, Rondo cultivar) and planktonic cultures of the following microorganisms: the symbiotic nitrogen fixer *Rlv* (effective strain RCAM 1022), the pathogen causing bacterial blight in peas *Psp* (strain 1845), and the pathogen causing potato ring rot *Cms* (strain 6889).

Bacterial cultures were grown for 3 days in a germinating chamber (in the range 23–25 °C) in

100-ml flasks filled with 50 ml of a liquid medium containing 10 g/l of yeast extract dialysate and 15 g/l of glucose, pH = 7.0. Microorganisms were grown without additional shaking. The density of the plankton culture of bacteria was determined using a tablet spectrophotometer Immunochem-2100 (High Technology Inc., USA) at a wavelength of 655 nm.

Pea seeds were sterilized and washed sequentially: 5 min in 94% ethanol, 5 min in 3% H_2O_2, and 5 min in a 5% solution of potassium permanganate. Following washing with sterile water, they were poured into a glass of hot water (60 °C) for 4 hours to allow the seeds to swell. Then, the seeds were germinated in sterile Petri dishes on moistened filter paper for 3 days in the dark at 23–25 °C. Seedlings with a root length of at least 35–40 mm were selected and washed with a sterile 0.01% solution of Nonidet (a nonionic detergent) to prevent the infusion of exogenous microflora, washed three times with sterile distilled water and inoculated with a culture of one of the bacteria in the stationary growth phase. The titer of the plankton culture of bacteria was 10^7 c/ml. The specimens were inoculated with bacteria for 5 minutes, after which the roots of the seedlings were separated from the pea, washed in a sterile 0.01% solution of Nonidet (a detergent) to remove loosely bound bacteria, then washed three times in sterile water.

*Determination of SAC and TAC activity, and cAMP concentration***.** To determine the activity of SACs and TACs, segments of seedling roots of length 22 mm were fixed in liquid nitrogen before enzymes were isolated following the previously developed method [9]. The roots were ground up in an isolation (germination) medium of the following composition: 0.02 M of phosphate buffer, pH = 7.2; 0.1 mM of theophylline (3',5'-cAMP of phosphodiesterase inhibitor); 1 mM of dithiothreitol (SH-group protector); 50 μg/ml of phenylmethylsulfonyl fluoride; 50 μg/ml of hydroxymercury benzoate; 1 μg/ml of leupeptin (protease inhibitor). The homogenate was centrifuged at 16,000 g for 20 min (Allegra 64 R) to remove fragments of cell walls and some organelles. The resulting supernatant was centrifuged at 105,000 g for 90 min (90 Sorvall Discovery SE).

The following parameters were analysed: the TAC activity of the sediment, which contains 70–75% of the plasmalemma [10], and both the SAC activity and the cAMP concentration in the membrane-free supernatant. Samples for cAMP determination were heated to 100 °C for 3 min to inactivate the enzymes.

The activity of both forms of AC was not measured directly but was considered to be directly proportional the cAMP concentration in the sample calculated per mg of protein/min. The cAMP concentration was determined by the enzyme-linked immunosorbent assay [11]. The cAMP concentration in cells was calculated per mg of protein; the protein in the sample was determined by Bradford's technique.

Determination of H_2O_2 concentration. The concentration of H_2O_2 was determined in the homogenate of segments of the root of pea seedling by the FOX method, based on the change in the colour of Xylenol orange [12]. This required a reagent consisting of two solutions: the first contained 25 mM of $FeSO_4$, 25 mM of $(NH_4)_2SO_4$, and 2.5 M of H_2SO_4, the second contained 125 μM of Xylenol orange and 100 mM of sorbitol. The solutions were mixed immediately prior to analysis in a ratio of 1:100, respectively. Then, the resulting mixture was added to the plant sample in the proportion of 1:10, respectively, and incubated for 30 minutes in the dark, after which the concentration of H_2O_2 was determined by absorbance at a wavelength of 595 nm using a tablet spectrophotometer M680 (Bio-Rad, Germany). The concentration of H_2O_2 was expressed in nmol per mg of protein. The analytical sample was a 1 g portion of the root of pea seedlings.

The effect of H_2O_2 on the activity of TACs and SACs in pea root cells. Plant samples of SACs/TACs containing 100–150 μg of protein/g of raw mass in 500 μl of the germination medium described above were made up to the following concentrations of H_2O_2: 100 nM, 260 nM, 2.6 μM and 26 μM. The reaction was allowed to progress for 30 minutes at 27 °C before being stopped by boiling in a water bath for 3 minutes.

Three biological replicates were performed in all experiments and the concentrations of cAMP and H_2O_2 were calculated from eight replicate analytical determinations. The experimental results were processed statistically in the SigmaPlot 12.3 program. The Student criterion (*t*) and the significance of differences between the experience variants (*P*) were estimated. The figures give the means and standard errors.

The work was performed with equipment located in the Common Use Center "Bioanalytica" using biological collections at the Common Use Center "Bioresource Center" of the Siberian Institute of Plant Physiology and Biochemistry within the Siberian Branch of the Russian Academy of Sciences (Irkutsk).

RESULTS AND DISCUSSION

The endogenous concentration of H_2O_2 and cAMP in the root of a pea seedling infected with a mutualist and pathogens. The concentration of H_2O_2 in the homogenate from uninfected pea root cells was found to be in the range of 200–270 nmol/mg of protein. The most pronounced increase in the concentration of H_2O_2 content was observed in pea seedlings infected with *Rlv*, while the smallest effect was associated with infection with *Cms*. In these samples, infection with *Rlv* increased the content of cAMP, while infection with *Psp* decreased the content of cAMP to 86%. It is interesting to note that infection with the non-specific pathogen *Cms* gave

rise to a cAMP concentration 5 times greater than in the control (Fig. 1). The means and standard errors (n = 3; $P \leq 0.001$) are given.

The effect of the exogenous concentration of H_2O_2 on the activity of TACs and SACs in vitro from the roots of pea seedlings infected with a mutualist and pathogens. In the control, ie. in the absence of biotic stress, the activity of SACs was dependent on the concentration of H_2O_2: SACs were slightly activated at 100 nM of H_2O_2 (110% of the control), there was virtually no observed effect at 260 nM, and there was a pronounced reduction of activity, down to 70% of the control, at micromolar concentrations (2.6 and 26 μM). The H_2O_2 had a similar effect on TAC activity: slightly reduced enzyme activity at 100 nM, and a maximum inhibitory effect at micromolar concentrations of H_2O_2 (Fig. 2). The means and standard errors (n = 3; $P \leq 0.001$) are given.

The effect of the exogenous concentration of H_2O_2 on the activity of TACs and SACs in vitro from the roots of pea seedlings infected with a mutualist and pathogens. In the control, i.e. in the absence of biotic stress, the activity of SACs was dependent on the concentration of H_2O_2: SACs were slightly activated at 100 nM of H_2O_2 (110% of the control), there was virtually no observed effect at 260 nM, and there was a pronounced reduction of activity, down to 70% of the control, at micromolar concentrations (2.6 and 26 μM). The H_2O_2 had a similar effect on TAC activity: slightly reduced enzyme activity at 100 nM, and a maximum inhibitory effect at micromolar concentrations of H_2O_2 (see Fig. 2). The means and standard errors (n = 3; $P \leq 0.001$) are given.

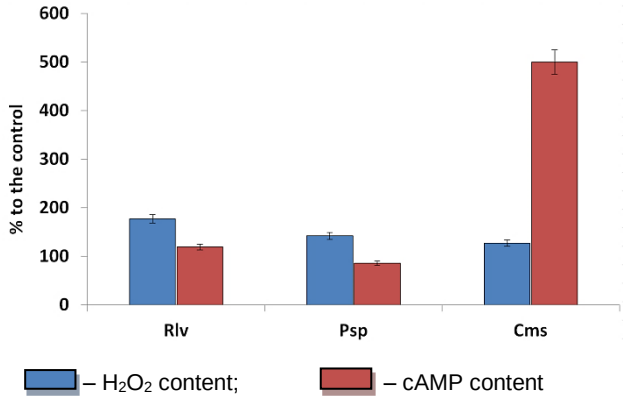

Fig. 1. Effect of infection with *Rhizobium leguminosarum* bv. *vicea* (*Rlv*), *Pseudomonas syringae* pv. *pisi* (*Psp*) and *Clavibacter michiganensis* ssp. *sepedonicus* (*Cms*) on the content of H_2O_2 (% of control) and cAMP (% of control) in the root of pea seedlings. The control was seedlings germinated in water

Рис. 1. Влияние инфицирования *Rhyzobium leguminosarum* bv. *vicea* (*Rlv*)/*Pseudomonas syringae pv. pisi* (*Ps*)/*Clavibacter michiganensis* ssp. *sepedonicus* (*Cms*) на содержание H_2O_2 (% к контролю) и цАМФ (% к контролю) в корне проростков гороха. Контролем служили проростки, инкубированные на воде

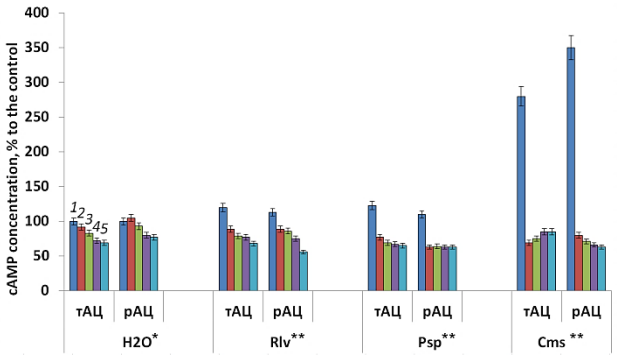

Fig. 2. Effect of various exogenous concentrations of H_2O_2 on TAC and SAC activity in the roots of pea seedlings infected with Rhizobium leguminosarum bv. vicea (Rlv), Pseudomonas syringae pv. pisi (Psp) and Clavibacter michiganensis ssp. sepedonicus (Cms): 1 – H_2O; 2 – 100 нМ H_2O_2; 3 – 260 нМ H_2O_2; 4 – 2,6 мкМ H_2O_2; 5 – 26 мкМ H_2O_2.
*sample 1 served as a control for samples 2–5;
***in vitro* adenylate cyclase samples isolated from uninfected pea seedlings served as a control for samples 1–5

Рис. 2. Влияние различных экзогенных концентраций H_2O_2 на активность тАЦ и рАЦ в корнях проростков гороха при инфицировании *Rhyzobium leguminosarum* bv. *vicea* (Rlv) / Pseudomonas syringae pv. pisi (Ps)/Clavibacter michiganensis ssp. sepedonicus (Cms): 1 – H_2O; 2 – 100 нМ H_2O_2; 3 – 260 нМ H_2O_2; 4 – 2,6 мкМ H_2O_2; 5 – 26 мкМ H_2O_2.
*Контролем для образцов 2–5 служил образец 1;
**контролем для образцов 1–5 служили образцы аденилатциклаз *in vitro*, выделенные из неинфицированных проростков гороха

Following a short-term, 5-min exposure to *Rlv*, the activity of SAC and TAC only slightly exceeded the activity in control specimens, 113 and 122%, respectively. The effect of infection with *Psp* had a similar effect on rhizobial infection. Following infection with *Cms*, the activity of SAC and TAC exceeded the activity in the control by 3 and 4 times, respectively (see Fig. 2).

The H_2O_2 concentration had a similar effect on the activity of SACs and TACs *in vitro, in Rlv*-inoculated cells of pea roots when compared with the control; however, a dose-dependent effect was observed under the following conditions: at 100 nM of H_2O_2 there was slightly reduced activity of both forms of the enzyme (90% of the control), and at 26 μM their activity was inhibited by 50–60% (see Fig. 2). Following infection with *Psp*, there was an equivalent effect reducing the activity of SACs and TACs (68–70% of the control) at all concentrations of H_2O_2 in the study. A different effect was observed in the case of infection of pea seedlings with a non-specific pathogen, *Cms*. The lowest concentration of H_2O_2 studied (100 nM) inhibited TAC activity the most, but had an activating effect on SAC activity. On the contrary, micromolar concentrations of H_2O_2 (2.6 and 26 μM) increased TAC activity and inhibited the activity of the soluble enzyme form (see Fig. 2).

It should be noted that the initial stages of pathogenic infection and legume-rhizobial symbiosis have many common features. One of the first nonspecific protective reactions of plant cells to infection is an increase in the concentration of intracellular H_2O_2 [13, 14]. Similarly, it has been reported that the nodulation of the lateral roots of the tropical semiaquatic plant *Sesbania rostrata* by the microsymbiont *Azorhizobium caulinodans* is accompanied by the generation of reactive oxygen species (ROS) initiated by the nod factor of the latter [15]. An increase in the concentration of H_2O_2 was detected when spring barley (*Hordeum vulgare* L.) was infected by a pathogenic strain of *Fusarium culmorum* [6]. We observed a similar effect in experiments after short-term inoculation of pea seedlings with *Rlv, Psp*, and *Cms* (see Fig. 1).

It is known that H_2O_2 in high concentrations can affect the lipid environment of membrane proteins, thereby affecting their functional activity [16, 17]. Millimolar concentrations of this compound can cause significant destruction of the structure of chloroplast membranes due to the very intensive peroxidation of membrane lipids [16]. In this experiment, lower, nanomolar and micromolar exogenous concentrations of H_2O_2 were studied; therefore, it is assumed that the observed effects on the activity of both forms of ACs are associated with conformational changes in the active centre of the enzyme localized outside the membrane (i.e. in the cytoplasm). This conclusion is supported by H_2O_2 concentration dependent changes in the activity of TACs and SACs, i.e. the observed dose-dependent effect (see Fig. 2). In addition, it is reported that H_2O_2 can oxidize some of the constituent amino acids (Arg, Pro, Lys, Met, Cys, Tyr, His) of enzymes leading to a change in the activity of the enzyme [18]. Although full amino acid sequencing of plant ACs has not yet been reported, the characterisation of the primary structure of protein fragments with AC activity has been reported [17, 18]. The proportion of amino acids which can be oxidized by H_2O_2 in the AC catalytic center (AtKUP71-100) in the multi-protein complex of K^+-dependent permease 7 (AtKUP7) [19] and the AC (AtLRRAC1) in a complex with the receptor (NBS-LRR) [20] was determined to be 27 and 28% of constituent amino acids, respectively [18]. The susceptible amino acids include the amino acid pair Lys-Arg, which is responsible for the conversion of ATP to cAMP in the AC catalytic center [21]. Thus, it is highly likely that H_2O_2 affects the catalytic functions of this enzyme. Further work to provide additional supporting data has been planned to study the kinetic parameters of ACs in the presence of H_2O_2.

It has already been shown that short-term infection of the roots of pea seedlings with *Rlv* and *Psp* led to a modulation of TAC and SAC activity and, consequently, to a change in the concentration of intracellular cAMP [3]. Thus, the enzyme activity had already been modified when the enzymes were isolated from the infected roots of pea seedlings. In our experiments, ACs isolated from the infected tissues had already been exposed to H_2O_2, as witnessed by an increased level of H_2O_2 in the cells of the root of pea seedlings (see Fig. 1). The observed slight decrease in the activity of ACs is probably due to the additive effect of endogenous and exogenous H_2O_2. It is interesting to note the different effect on TACs and SACs in the experiments treated with *Rlv*+H_2O_2 and *Psp*+H_2O_2, viz. similar activity levels of TACs were observed, whereas activity levels of SACs differed slightly. It can be assumed that in a very short time (5 min) the activity of TACs could be modified by nonspecific virulence factors of *Rlv* and *Psp* such as some polysaccharides and surface proteins of bacteria that are similar in nature to those of gram-negative bacteria [22]. At the same time, SAC activity can be modulated only indirectly, probably with the involvement of other second messengers. For example, the activation of SACs from beet cell vacuoles by calcium ions has been reported [9]. It can be assumed that a local increase in the concentration of H_2O_2 not only leads to a modulation of the activity of this enzyme form (see Fig. 2), but also causes a change in the concentration of other signaling molecules, and in particular, calcium ions. This is supported in the literature by reports of peroxide-dependent calcium ion channels found in many plant species, including pea [23]. It is obvious that the dynamics of the intracellular calcium pool is specific to the type of microorganism causing the infection. This dynamics is dependent on the nature of various types of ion channels, including activated cAMP. The lack of sensitivity of SACs to H_2O_2 after Psp infection may be related to this.

The completely different effect of infection with *Cms* followed by the addition of exogenous H_2O_2 on the activity of TACs and SACs is very interesting. *Cms* has a wide range of exopolysaccharides, which is a principal reason for its virulence. These exometabolites have a fairly acidic nature [24], which can lead to temporary, non-specific acidification of the extracellular space in plants [25]. A sharp decrease in the pH of the pericellular environment can lead to a change in the electrochemical potential of the cell membrane [26] further inducing change in the pool of various second messengers, including H_2O_2. It can be assumed that this is the principal cause of the "preliminary" modification of the activity of AC *in vivo*, which subsequently affected the sensitivity of the AC to additional concentrations of H_2O_2 *in vitro* (see Fig. 2).

CONCLUSION

The activity of transmembrane and soluble adenylate cyclases from the root cells of pea seedlings can be dose-dependently modulated by H_2O_2. The biotic stress phenomenon may be additionally affected by dosing with hydrogen peroxide.

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Contribution

Olga V. Kuzakova, Lidia A. Lomovatskaya, Anatoly S. Romanenko, Alena M. Goncharova carried out the experimental work. The authors on the basis of the results summarized the material and wrote the manuscript. All authors have equal author's rights and bear equal responsibility for plagiarism.

Критерии авторства

Кузакова О.В., Ломоватская Л.А., Романенко А.С., Гончарова А.М. выполнили экспериментальную работу. Авторы совместно обобщили результаты, написали рукопись, имеют на статью равные авторские права и несут равную ответственность за плагиат.

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