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RESEARCH PAPERS

Ethylene in the Proliferation of Cultured Plant Cells: Regulating or Just Going Along?

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Abstract—Ethylene, being one of five classical plant phytohormones is involved in regulation of numerous physiological processes. There are contradictory data about the effect of ethylene on the cell growth and division; although it is accepted that in culture flasks, the content of ethylene rises to a few tens of μL/L and production of ethylene is associated with the periods of active growth of the cells in vitro. We revealed a strong correlation ($r = 0.96$) between ethylene production and specific rate of dry weight accumulation in suspension cell cultures of *Ajuga turkestanica*, heterotrophic and mixotrophic strains of *Arabidopsis thaliana*, *Beta vulgaris*, *Euonymus maximoviczianus*, *Medicago sativa*, *Panax ginseng*, and *Triticum timopheevii*. In heterotrophic cell culture of *A. thaliana*, the peaks and general shape of the curves describing dynamics of ethylene production, the number of S-phase cells, and specific rate of increase in cell number coincided in log phase and in the phase of growth deceleration. Pretreatment of subculture inoculum with 100 μL/L ethylene caused doubling of S-phase cell number after 3-h-long culturing in fresh nutrient medium. It was found that exogenous ethylene affects the number of S-phase cells only when the level of endogenously produced ethylene is low.

Keywords: Arabidopsis thaliana, S-phase, cell cycle, cell culture, cell proliferation, growth, ethylene production
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INTRODUCTION

Since the beginning of the 20th century when disturbance of normal growth in plants exposed to extremely low concentrations of gaseous ethylene was shown [1], it was found that its influence on plants is diverse. Ethylene is one of five classical phytohormones; it regulates termination of seed dormancy and transition to germination, cell elongation, formation of root hairs, growth of reproductive organs and determination of sex of flowers, senescence and abscission of leaves and flowers, maturation and abscission of fruits, and responses to pathogens and abiotic stress factors [2, 3].

Molecular mechanisms of ethylene action have been and still remain a matter of numerous studies during the three recent decades. Basic data were obtained as a result of large-scale genetic works dealing with the mutants of a model plant *Arabidopsis thaliana* [4, 5]. Identified key components participating in ethylene reception and its signal transduction allowed to offer a linear pathway including the binding of ethylene to receptor histidine kinases, modification of their conformation, and inactivation of receptor complexes with CTR1 (Ser/Thr protein kinase similar to protein kinases of Raf family). This causes dephosphorylation of protein EIN2 (protein similar to metal-ion transporter Nramp), cut off of its C-terminus domain that is transferred to the nucleus and initiates transcriptional response of ethylene-dependent genes by means of step-by step activation of transcription factors belonging to EIN3/EIL1 and ERF families [3, 6, 7].

However, the present pattern of ethylene signaling pathway acquired important elements: intervention in its different sections of the modules of mitogen-activated protein kinases, involvement of proteasome-mediated degradation proteins involved in signaling pathway, and ethylene-regulated availability of receptor/CTR1 complexes [8–11]. This suggests that fine tuning of ethylene perception and transduction of ethylene signal, as well as availability of alternative signaling pathways, is very important for plants, which urges the researchers to more attentively look into this issue.

The role of ethylene in the regulation of growth and cell divisions also needs investigation. Although the evidence about ethylene as an inhibitor of cell elongation was formulated long ago, there are numerous examples when ethylene stimulated growth, which as

Abbreviations: CC—cell cycle; EdU—5-ethynyl-2'-deoxyuridine; ERF—ethylene response factors; PBS—phosphate buffered saline; SH—Schenk and Hildebrandt medium.

a rule depended on phytohormone concentration, plant species, and environmental conditions [12, 13].

The role of ethylene in the control over cell proliferation is not unambiguous. On the one hand, ethylene inhibited replication of nuclear DNA and cell divisions in pea seedlings [14] and induced programmed cell death at certain periods of the cell cycle (CC) [15]. On the other hand, ethylene caused endoreduplication [16], stimulated division of stem cells of quiescent center in root meristem of *Arabidopsis* [17], cambial cells of poplar [18], and the cells of quiescent center in maize roots after the excision of the root tip [19].

Investigations of the effect of any agents on CC of plants, including the role of ethylene in the regulation of cell divisions and molecular mechanisms of its interaction with known components governing CC, are most informative in the experimental objects with intense proliferation of the cells and that are free of complicated interactions between the tissues and devoid of complex ontogenetic programs. These conditions are fulfilled in actively growing suspension cell cultures that are widely used in experimental plant biology [20].

It is important that depending on plant species and growing conditions, the cells and tissues cultured in vitro can produce ethylene that accumulate in culture vessels at a concentration of up to a few tens of μL/L [21, 22], whereas physiological effects of ethylene have been shown at concentrations by two or three orders of magnitude lower [1, 2]. Moreover, it was shown that production of ethylene is associated with periods of active growth of cultured cells [21, 23]. Therefore, a high level of ethylene must be consistent with cell proliferation in vitro. However, the results of early investigations and conclusions made by the researchers are controversial: some of them argue that ethylene is a side product of rapid growth and does not participate in initiation and maintenance of division of plant cells in vitro; the others report about opposite effects of exogenous ethylene on the growth of callus and suspension cultures or a lack of effect even at a very high (approximately 20 mM) concentration of the phytohormone [21–23].

Thus, the role of ethylene in the regulation of proliferation of plant cells in vivo and in vitro is still unknown. This urged us to tackle again the relationships between growth parameters of cultured cells and production of ethylene and look into the effect of exogenous ethylene on the number of cells in the phase of nuclear DNA replication as an indicator of active passage through CC.

MATERIALS AND METHODS

The objects of investigation were suspension cell cultures from the All-Russia Collection of Cultivated Cells of Higher Plants: (1) *Beta vulgaris* L., strain 2*n*; (2) *Triticum timopheevii* (Zhuk.) Zhuk.; (3) *Panax ginseng* C.A. Mey., strain PgL1 NB; (4) *Medicago sativa* L.; (5) *Dioscorea deltoidea* Wall., strain DM-05, line 3; (6) *Ajuga turkestanica* (Regel) Briq., line1. In addition, we used the strains designed by A.V. Nosov and A. A. Fomenkov: (7) heterotrophic strain of *Arabidopsis thaliana* (L.) Heynh. of wild type (ecotype Columbia, Col-0) [24]; (8) mixotrophic strain of *A. thaliana*, and (9) suspension cell culture of *Euonymus maximoviczianus* Prokh. The cells of strains 1, 2, and 7–9 were cultured on Schenk and Hildebrandt (SH) medium with 3% sucrose (1.5% for strain 8), 1 mg/L 2,4-D, and 0.1 mg/L kinetin; cells of strains 3–6 were cultured on MS medium with 3% sucrose, 1 mg/L 2,4-D and 0.1 mg/L kinetin (for strains 4 and 5), 2 mg/L NAA and 1 mg/L BAP (strain 3), 1 mg/L 2,4-D, 1 mg/L IAA, and 0.2 mg/L BAP (strain 6). The cells were cultured in 250–300-mL glass flasks capped with aluminum foil and kraft paper (strain 4 capped with cotton-wool plugs) in the dark (strain 8 was permanently illuminated with luminescent lamps at light intensity of 200 μmol photons/(m^2 s)) at a temperature of 26°C and constant agitation (120 rpm). Subculturing period was 10 days for strains 7 and 8, 14 days for strains 1–6, and 20 days for strain 9.

In order to determine growth parameters of cell suspension during subculturing, we periodically collected three flasks each where fresh weight and dry weight (after drying at 37°C for 3 days) were determined.

The number of cells of heterotrophic strain of *A. thaliana* was estimated by the number of protoplasts. For isolation of protoplasts, we mixed equal volumes of cell suspension and the solution containing SH medium macronutrients, 0.8 M sorbitol, 8 mM $CaCl_2$, 25 mM Mes/KOH (pH 5.7), 2% cellulase Onozuka R10 (Kinki Yakult, Japan), 0.3% pectinase Macerozyme R10 (Kinki Yakult), and 0.8% hemicellulase Driselase (Fluka, United States). Protoplasts were isolated at 26°C in the course of incubation of suspension on a shaker (100 rpm) for 1.0–1.5 h. Protoplasts were counted in a Fuchs-Rosenthal chamber.

Viability was estimated by the number of cells unstained with 0.02% aqueous solution of Erythrosin B (Sigma-Aldrich, United States).

The number of cultured cells of *A. thaliana* in S-phase of CC was determined by incorporation in replicating DNA of thymidine analog 5-ethynyl-2'-deoxyuridine (EdU, Invitrogen, Life Technologies, United States) according to the method we described earlier [25]. During a standard growing cycle or after the end of treatment with exogenous ethylene lasting for 1 day or longer, we took the samples of the cells (5–6 mL), added 20 μM EdU, and incubated for 1 h in the dark at 26°C on a shaker (for a hour exposure to ethylene, the procedure of incubation with EdU is given

below). To terminate incorporation of EdU, 200 μM thymidine was added. The cells were fixed in 4% Formalin in phosphate buffered saline (PBS) with 0.1% Triton X-100 or, otherwise, protoplasts were isolated from the cells (see determination of cell number) and fixed with cold methanol. Incorporation of EdU was detected by the reaction with azide Alexa Fluor 488, the nuclei were stained with 100 ng/mL DAPI (4',6-diamidino-2-phenylindole), temporary preparations were made and examined under an Axio Imager Z2 fluorescence microscope (Zeiss, Germany) [25]. Images were processed using an AxioVision 4.8 program. Total number of nuclei examined on several preparations was no less than 5000 with the number of S-phase cells < 3% and approximately 2000 with the number of S-phase cells >3%.

Production of ethylene and determination of ethylene, carbon dioxide and oxygen were described earlier [26]. Ethylene was produced as a result of decomposition of 2-chloroethylphosphonic acid under basic conditions. Quantification of ethylene was performed using a Tzvet 106 gas chromatograph (Russia) equipped with a flame ionization detector. Hydrocarbons were concentrated on a Porapak N column (80–100 mesh, 70 × 4 mm) (Supelco, United States) at –30°C. After desorption at 50°C, ethylene was assayed using a Porapak N column (80–100 mesh, 3 m × 2 mm). The content of carbon dioxide and oxygen was checked by the method of gas adsorption chromatography. The system of concentration much improved sensitivity of the method, because it makes it possible to use the whole gaseous phase of the bottle and reduce the duration of incubation of the cells under gas-impermeable plugs therefore preserving the level of oxygen necessary for life and activity of oxidase of 1-aminocyclopropane-1-carboxylic acid.

In order to estimate the rate of ethylene production, in different periods of subculturing, we took 3–5 mL of cell suspension each from culture vessels and transferred the samples to 15-mL glass bottles. These bottles were placed under temperature-controlled conditions (26°C) on a shaker (100 rpm) for 10-min-long airing; then they were plugged with sealing rubber stoppers (Suba-Seal septa red rubber, Sigma-Aldrich) and incubated for 0.5–1.0 h at 26°C on a shaker in the dark. The bottles with the medium free of cells were treated in the same way; their gas phase was used as a reference for the content of ethylene in the air. Then, 1–2 mL of gas medium was taken from the bottles with a syringe for determination of O_2 and CO_2 and the remaining gas was used for the determination of ethylene. Special attention should be paid to adhering to temperature regime and necessity of stirring in the course of manipulations with the cells, because production of ethylene depends on these parameters. In order to determine composition of gaseous medium in the flasks with cultured cells, we punctured the foil caps of the flasks with a long-needle syringe and extracted the samples of gas (10 mL); 1–2 mL aliquots were used for analysis. Gas composition was also checked in gas bell jars and airtight containers, where the cells were cultured in the atmosphere with exogenous ethylene.

Culturing the cells of *A. thaliana* with exogenous ethylene. When the cells were exposed to ethylene for more than two days, suspension was grown in 100-mL Erlenmeyer flasks. One-half milliliter of cell suspension was applied to 25 mL of SH medium. The flasks were covered with a single layer of sterile dense chromatographic paper, placed on the table made of organic glass, covered with a 20-L gas bell jar, and sealed. Through tubing attached to the fitting coming out of the table, ethylene (20 μL/L or 100 μL/L) was applied to the bell jar. Control bell jar was filled with air. The whole constructions were placed on a shaker (26°C, darkness). When the cells were exposed to ethylene for two days, 6-L gas bell jars were used. Twenty-four-hour-long exposures were conducted in 2.6-L polypropylene containers equipped with tubings and airtight covers with silicon seal. In this case, cell suspension was grown in broad 100-mL beakers covered with dense chromatographic paper.

In the experiments with pretreatment of heterotrophic cell culture with ethylene before its inoculation to fresh medium, 100-mL beakers with 10-d-old suspension were placed in containers near a broad crystallizer containing 50 mL of SH medium with 20 μM EdU. The container was closed and ethylene (100 μL/L) was applied. After 5 h exposure with ethylene, the container was opened; 1 mL of 10-d-old suspension was quickly transferred to crystallizer with the medium, then the container was closed and ethylene was fed again. The container was placed on a shaker for 3 h (26°C, darkness). Simultaneously, 1 mL of cell suspension was applied to 250-mL flasks with 50 mL of SH medium and 20 μM EdU and cultured under standard conditions. During 4 h, we recorded production of ethylene by the cells and fixed material for determination of the number of S-phase cells.

The figures show the means of three replications and their standard errors.

RESULTS AND DISCUSSION

In the course of subculturing, suspension cell cultures of all the tested strains released ethylene that accumulated in the gas phase of the flasks (table). It is interesting that the content of ethylene was from one to three orders of magnitude higher than in the air and greatly varied in different cell cultures. Other researchers also observed that ethylene content varied in a wide range depending on the type of cultured cells and tissues and reported that more intense production of ethylene was often associated with the periods of

Content of ethylene in gas phase in the flasks with suspension cell cultures

Suspension cell culture	Content of ethylene, nL/L	μ_{max}, day^{-1}
Beta vulgaris	73	0.47
Triticum timopheevii	83	0.30
Panax ginseng	10	0.16
Euonymus maximoviczianus	42	0.09
Dioscorea deltoidea	31	0.18
Arabidopsis thaliana (heterotrophic strain)	3420	0.86
A. thaliana (mixotrophic strain)	2970	0.69

Content of ethylene in the air was 5 nL/L. μ_{max}, day^{-1} is maximum specific rate of dry weight accumulation.

active growth [21–23]. However, based on available data, it is impossible to reveal a correlation between the rate of growth of cultured cells and production of ethylene because of the lack of growth characteristics and on account of specific methods of recording the release of ethylene. There is no doubt that the content of ethylene in the gas phase must depend on its production by cultured cells and if the release of ethylene is related to the rate of growth, the level of ethylene should also depend on growth rate. The table shows that the greatest content of ethylene was found in gas phase of suspension cultures of *A. thaliana* with the highest rate of growth. However, the content of ethylene in the gas phase also depended on air tightness of the flasks, the amount of biomass, and the volume of the medium and its composition.

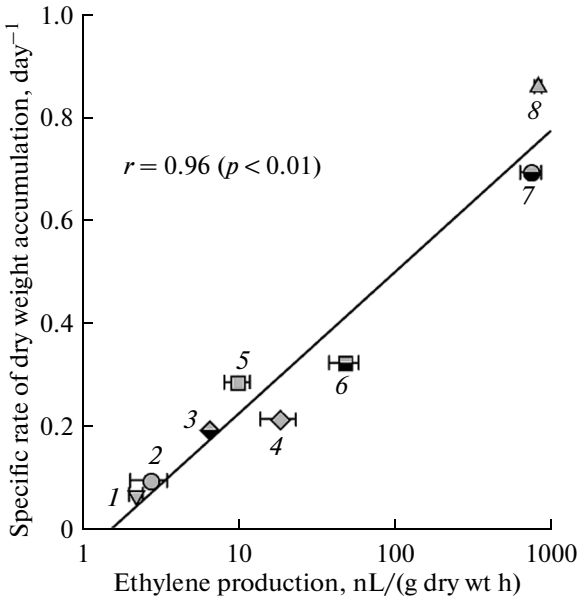

Fig. 1. Relationship between specific rate of dry weight accumulation and production of ethylene in suspension cell cultures. (*1*) *Panax ginseng*, (*2*) *Euonymus maximoviczianus*, (*3*) *Beta vulgaris*, (*4*) *Medicago sativa*, (*5*) *Triticum timopheevii*, (*6*) *Ajuga turkestanica*, (*7*) *Arabidopsis thaliana* (mixotrophic strain), and (*8*) *A. thaliana* (heterotrophic strain). Abscissa is represented on a log scale.

Therefore, we find it more correct to consider a possible relation between ethylene production by the cultured cells and specific growth rate. Instead of maximum specific rate, it is advisable to use a particular specific rate related to the time interval when ethylene production was determined. In addition, production of ethylene should be calculated on dry weight unit basis, because the cultures differ in the content of water. The obtained data showed that, irrespective of the strain, there exists a strong correlation between production of ethylene and specific rate of dry weight accumulation (Fig. 1). However, the revealed relationship does not make it possible to positively suggest causality between these parameters. For a more detailed analysis, we chose the most rapidly growing heterotrophic suspension cell culture of *A. thaliana*. Throughout the cycle of growing, we determined growth characteristics, production of ethylene, and the number of cells being in the period of nuclear DNA replication (S-phase cells).

Right after inoculation of the cells to a fresh nutrient medium, production of ethylene sharply rose (Fig. 2, curve *1*); then it declined, grew on the first day, and attained maximum in the middle of log phase of growth

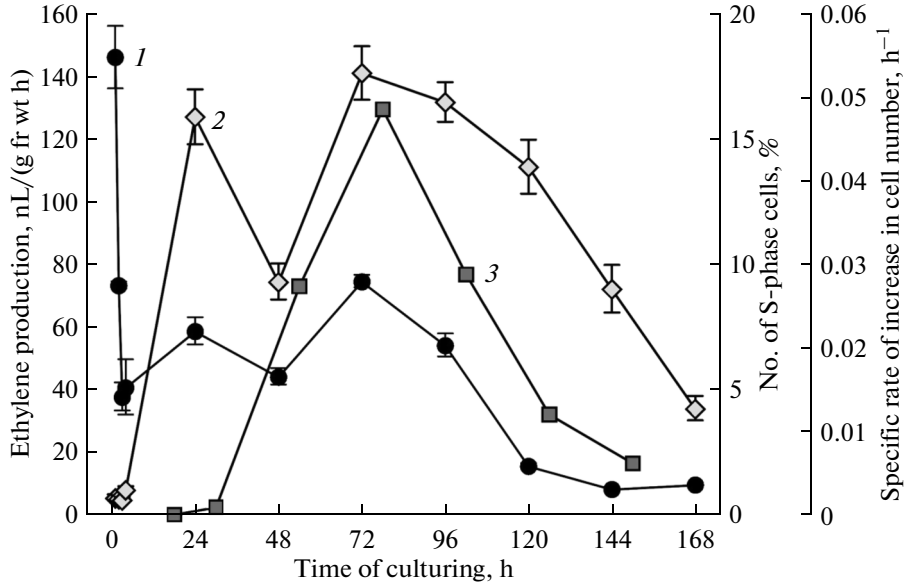

Fig. 2. (*1*) Time course of ethylene production, (*2*) the number of S-phase cells, and (*3*) specific rate of increase in cell number in the growth cycle of heterotrophic suspension cell culture of *A. thaliana*.

(72 h). It is important that, starting from the beginning of log phase (48 h) and further throughout the cycle of growing, we observed coincidence of the peaks and general shape of the curves describing dynamics of ethylene production, the number of S-phase cells, and specific rate of increase in cell number (Fig. 2, curves *1*–*3*).

The first peak of S-phase cells, in percentage terms comparable to the peak observed on the 72nd hour, was observed on the first day when absolute number of cells essentially did not change from the time of their inoculation to fresh nutrient medium. This peak is characteristic of a well-known phenomenon of partial synchronization of CC at the beginning of subculturing when the cells receive a new batch of carbohydrates, nitrates, and phosphates, which was long ago realized in the patterns of CC synchronization by means of elimination of certain nutrient components with their subsequent addition [27]. The fact that the first peak of S-phase cells coincided with a rise in ethylene production at a term of 24 h can point to participation of ethylene in realization of events of partial synchronization at the beginning of subculturing. It is known that carbohydrates stimulate synthesis of ethylene, and the peak of its production by the discs from tobacco leaves (upon the addition of sucrose in the presence of auxin) falls at 24 h [28].

Let us return to the log phase of growth. Proceeding from the maximum specific rate of increase in the number of cells (μ_{max} = 0.048 h^{-1}), their doubling time is 14.4 h. If we assume that ethylene is a trigger (not a result) of cell proliferation, then a graph of "ethylene production" against "specific rate of increase in cell number" should be plotted with the latter parameter calculated for 12-h-long time interval after the point where ethylene production was determined. Actually, in that case, correlation between ethylene release and specific rate of increase in the number of cells is rather strong (Fig. 3a). If we calculate the value of μ for 12-h-long time interval preceding the point where production of ethylene was determined, correlation collapses (Fig. 3b). This indirectly indicates that ethylene synthesis precedes proliferation of the cells.

Now let us turn to the beginning of growing cycle. As was noted, a burst of ethylene production during the first hour of culturing was followed by its rapid recession (Fig. 4, curve *1*). By the fourth hour, the number of S-phase cells began growing (Fig. 4, column *2*). Activation of ethylene release can be accounted for by both physical manipulations with the cells (pipetting) and renewal of the medium causing changes in pH, osmolarity, and ion composition. It is known that, in response to mechanical injury, production of ethylene in plants increases within several minutes [29]. In case of *A. thaliana* cell suspension, such a short-term but high level of ethylene may be a signal that the cells should progress from G1-phase of CC to S-phase. It was shown recently that excision of apical meristem of the main shoot unconditionally resulting in production of wounding ethylene quickly activated expression of the gene of transcription factor ERF114 (otherwise called EBE or ERF BUD ENHANCER) that activates transcription of D-type cyclin gene (*CYCD3;3*)—a necessary participant of regulation of G1/S transition in CC [30]. Probably, such a sequence of events also occurs when the cells are inoculated to the fresh medium.

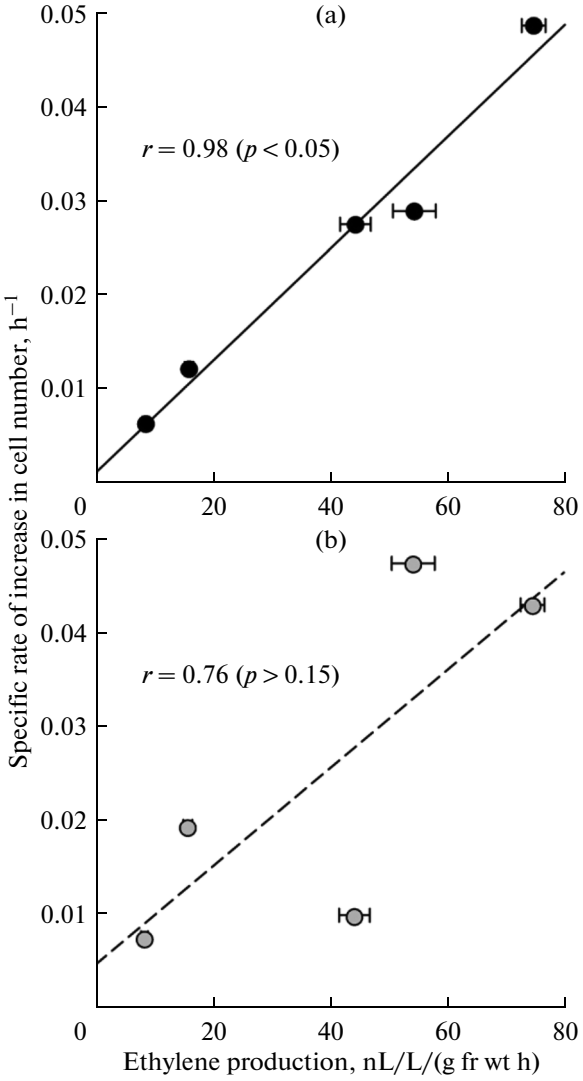

Fig. 3. Relationship between specific rate of increase in cell number and production of ethylene in heterotrophic suspension cell culture of *A. thaliana*. (a) specific rate of increase in cell number calculated for 12-h-long interval after the point when ethylene production was determined; (b) specific rate of increase in cell number calculated for 12-h-long interval prior the point when ethylene production was determined.

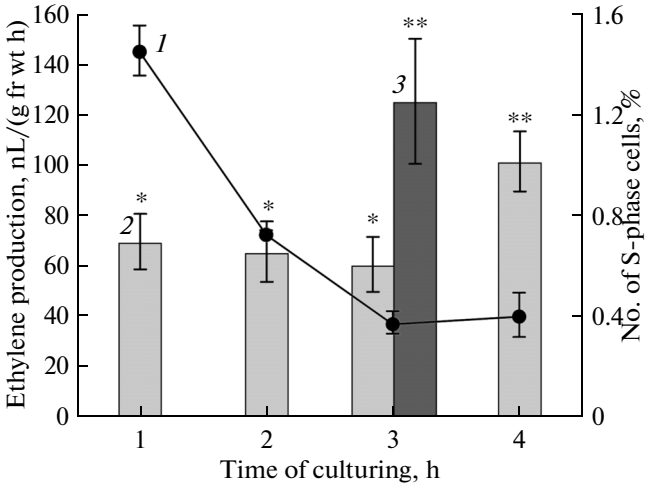

Fig. 4. Time course of ethylene production and the number of S-phase cells within the next few hours after inoculation of the cells of heterotrophic strain of *A. thaliana* to fresh nutrient medium. (*1*) ethylene production, (*2*) number of S-phase cells in control material (air), and (*3*) number of S-phase cells after pretreatment of inoculum with ethylene (100 μL/L) for 5 h and cell culturing after inoculation in the atmosphere with 100 μL/L ethylene. Equal number of asterisks designates the lack of reliable differences at $p < 0.05$.

Let us assume that ethylene is one of the factors participating in regulation of cell transition from phase G1 to phase S of CC and is necessary for the maintenance of their active proliferation; then these processes can be probably stimulated by exogenous ethylene. After pretreatment of 10-d-old cells with ethylene at a concentration of 100 μL/L during 5 h, they were inoculated to fresh medium and cultured at the same concentration of ethylene in the atmosphere. As a result, in 3 h the number of S-phase cells was two times greater than in the culture kept in the air (Fig. 4, column *3*; Fig. 5, type of treatment *1*). If culturing of cell suspension in gas medium started with 20 μL/L or 100 μL/L ethylene applied right after inoculation, there was no effect either on the first or on the fourth day (Fig. 5, types of treatment *2* and *3*). Similar result was obtained when 100 μL/L ethylene was applied from the first to the third day (Fig. 5, type of treatment *4*). A slight increase in the number of S-phase cells was observed in case of their culturing in the atmosphere with 100 μL/L ethylene during 24 h starting from the fourth day (Fig. 5, type of treatment *5*). If cell culture was exposed to ethylene starting from the fifth day (Fig. 5, type of treatment *6*), the index of S-phase cells considerably rose on the ninth day.

It should be noted that the culturing of cell suspension in a gas medium with ethylene right after inoculation in practice means that ethylene was applied 1 h after inoculation, because it takes time to close culture vessels, seal gas bell jars, and apply ethylene. It is possible that ethylene whose production is induced in the course of inoculation of the cells to fresh nutrient medium occupies all the vacant binding sites on ethylene receptors; therefore, exogenous ethylene cannot evoke a response at the moment. However, if we compare curves *1* in Fig. 2 and Fig. 5, it becomes evident that exogenous ethylene is efficient when production of endogenous ethylene is low. At concentrations used in this work, ethylene did not reduce viability of cell culture. Moreover, cell culturing with ethylene throughout the whole growing cycle led to elevation of growth index [24].

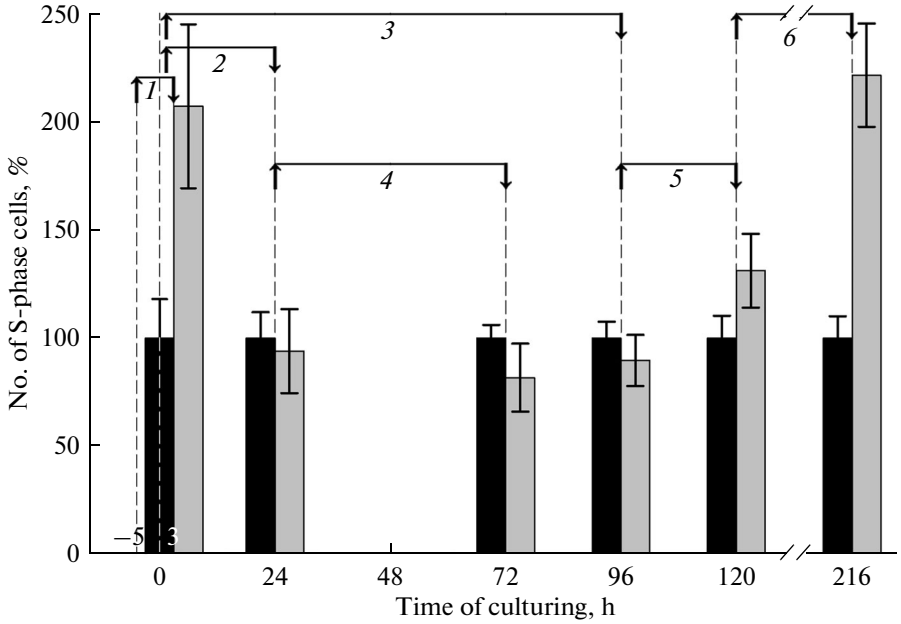

Fig. 5. Influence of exogenous ethylene on the number of S-phase cells depending on the time of treatment with ethylene of heterotrophic suspension culture of *A. thaliana* during the growth cycle. (*1*) pretreatment of the cells 5 h prior to inoculation, analysis 3 h after inoculation; (*2*) culturing in gas medium with ethylene for 24 h after inoculation; (*3*) culturing in gas medium with ethylene for 96 h after inoculation; (*4*) beginning of culturing in gas medium with ethylene 24 h after inoculation, analysis in 72 h; (*5*) beginning of culturing in gas medium with ethylene 96 h after inoculation, analysis in 120 h; and (*6*) beginning of culturing in gas medium with ethylene 120 h after inoculation, analysis in 216 h. Upward arrow designates the beginning of incubation in gas medium with ethylene. Downward arrow designates the time of fixation for counting S-phase cells. Black columns—control (untreated cells); grey columns—percent of control level.

Thus, the results obtained in this work give grounds to consider ethylene not as a companion of proliferation of cultured plant cells but as an active regulator of cell proliferation whose mechanism is yet to be discovered.

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