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COMPARATIVE ASSESSMENT OF THE EFFECT OF GIBBERELLIC AND SALICYLIC ACIDS ON THE GROWTH AND BIOCHEMICAL PARAMETERS OF *Phaeodactylum tricornutum*

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ABSTRACT

The research of the effect of gibberellic (0.4 - 3.8× 10^{-8} mol.L^{-1}) and salicylic (0.4 - 3.8× 10^{-5} mol.L^{-1}) acids, in a wide range of their concentrations, on the growth indicators and biochemical composition of the cumulative culture of the microalgae *Phaeodactylum tricornutum* was carried out. It was determined that salicylic acid in a concentration of 0.4 × 10^{-5} mol stimulated cell growth by 184.6%, and gibberellic acid at a concentration of 0.39× 10^{-8} mol by 181%, compared to the control. The effect of gibberellic acid during the experiment was expressed in the inhibition of protein accumulation in the culture, compared with the control. The use of salicylic acid led to a greater accumulation of protein in the culture than when using gibberellic acid. It was shown that salicylic acid had a positive effect on the accumulation of carbohydrates on day 9 and gibberellic acid on day 14 of culture. Gibberellic acid had no effect on the accumulation of lipids in the culture of microalgae. Under the action of salicylic acid for 14 days of cultivation, the lipid content increased by 18.5%, compared with the control. There were no quantitative differences in the content of chlorophyll when using two phytohormones. In this study, the optimal concentrations of gibberellic and salicylic acids for linear growth rate and the highest production of protein and carbohydrates for *Phaeodactylum tricornutum* were determined. Position, depending on the stage of microalgae growth, is noted.

KEYWORDS: cultivation; gibberellic acid; *Phaeodactylum tricornutum*; salicylic acid

INTRODUCTION

Marine diatoms play a key role as a food object in invertebrate mariculture, due to their high growth rate and high nutritional value. A small number of microalgae strains are usually cultured for use as feed, based on practical considerations of ease cultivation, physical characteristics of the cells, composition of the nutrient medium, and digestibility. The greatest demand for live feed is characteristic of the bivalve mollusks cultivation, which are filter feeders throughout their lives. Various microalgae species or combinations of microalgae are used as feed for both juvenile mollusks and in breeding stock maintenance (Helm *et al.,* 2004).

As confirmed by numerous studies, microalgae are a type of potential ingredient that can be used to enhance the nutritional value of foods (Caporgno & Mathys, 2018). The high nutritional potential is due to its high nutritional value and potential health benefits due to its high content of protein with a balanced amino acid composition, vitamins, minerals, short- and long-chain polyunsaturated fatty acids, carotenoids, enzymes and fiber (Batista *et al.,* 2019).

Phaeodactylum tricornutum is the only species of its genus, from marine diatoms, which is characterized by the accumulation of a high lipid content. This largely determines its high nutritional value.

The combination of heterotrophic ability and the ability to produce high value products in significant quantities is important for large-scale commercial exploitation of microalgae. The unicellular diatom alga *Ph. tricornutum* naturally accumulates high levels of EPA and has recently emerged as a potential source for its commercial production. It has a fast growth rate and can accumulate lipids in the form of triacylglycerols up to 30% of dry cell weight.

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The sea diatom alga *Ph. tricornutum* is an excellent candidate for high quality lipid production due to its fast growth cycle, sequenced genome and high lipid content. However, its inability to grow under heterotrophic conditions limits its potential as a commercial strain.

Transformation of this alga by a single gene encoding a glucose transporter (glut1) has shown that under heterotrophic conditions, in the absence of appropriate carbon transporters and metabolic pathways, some strains cannot use exogenous sugars for growth (Hamilton *et al.,* 2016). The physiological and biochemical characteristics of microalgae significantly depend on the conditions of their cultivation. The production of microalgae biomass depends not only on the amount and composition of primary nutrients, such as nitrogen and phosphorus, but also on the content of metals and vitamins in the environment of cations (Dragone *et al.,* 2011; Rasdi & Qin, 2015; Burch *et al.,* 2021).

In addition, the nutrients Rasdi restriction, especially of phosphorus and nitrogen, inhibits cell growth and protein synthesis. It also increases the production of carbohydrates or lipids (Rasdi & Qin, 2015). The growth of microalgae biomass significantly depends on the light conditions that affect the composition of fatty acids (Sharma *et al.,* 2020).

However, changing the composition of primary nutrients is not the only way to increase the productivity of microalgae cultures. In recent years, phytohormones of various chemical nature have been widely used to improve the growth of microalgae. It is known that phytohormones promote cell growth in low concentrations and inhibit their growth in high concentrations (Xu *et al.,* 2017). The mechanisms of action of most phytohormones are still being investigated. Previous researches do not always show the effective concentration of phytohormones, estimated by the growth of biomass and the yield of the main nutrient components.

Salicylic and gibberellic acids are important plant hormones that play a crucial role in protecting plants from pathogenic infection and stress (Rodas-Junco *et al.,* 2020; Shahzad *et al.,* 2021). Salicylic acid (SA) - phenolic compound containing an aromatic benzene ring with one or more hydroxyl groups formed as a secondary metabolite in plants and some microorganisms (Van Hung, 2016). In plants, phenolic compounds act as regulators of various biochemical and physiological processes (Wallis & Galarneau, 2020). The quantitative content of SA, between different plant species even within the same family can reach 1000 times (Mishra & Baek, 2021). SA influences plant growth and development by regulating various processes such as photosynthesis, respiration, vegetative growth, seed germination, flowering, and senescence (Butselaar & Ackerveken, 2020).

Gibberellic acid (GA) are the most important endogenous hormones found in plants and fungi, controlling plant development by regulating several physiological mechanisms. GA stimulate stem, root, and leaf growth, flowering, fruit senescence, seed germination, or dormancy (Basra, 2000). Their mechanism of action is determined by the transcription of genes involved in cell division during plant growth. In addition, they stimulate the expression of hydrolytic enzymes that convert starch to sugar, chlorophyll synthesis and degradation, and nitrogen metabolism, all of which influence overall plant growth (Miceli *et al.,* 2019).

The biochemical and physiological effects of GA vary with relative concentrations and plant response at different growth stages (Hedden & Sponsel, 2015).

The mechanisms of most phytohormones action are still being elucidated. There is limited information on the action of phytohormones under practical cultivation conditions or throughout the life cycle of microalgae. Previous studies do not always show the effective concentration of phytohormones as assessed by biomass growth and content of major nutrients. Thus, the effects of SA - 0, 1, 2, 6, 10, 14, 18, 30 mg L^{-1} and GA 0, 0.15, 1.50, 2.00, and 20.0 mg L^{-1} on the accumulation of oleinogenic acids in *Ph. tricornutum* culture were evaluated (Chu *et al.,* 2019).

This research aimed to (1) determine the optimal concentrations of phytohormones - gibberellic and salicylic acids, for the growth of *Ph. tricornutum* culture, and (2) evaluate their effect on the biochemical composition of microalgae.

MATERIALS AND METHODS

As source material for cultivation, a laboratory algologically pure culture of *Ph. tricornutum*, from the collection of the Far Eastern State Technical Fisheries University, was used. The initial concentration of *Ph. tricornutum* was 1.96 million cells for salicylic acid, and 1.87 million cells for gibberellic acid evaluation. Conical heat-resistant glass flasks with a volume of 1 liter were used as cultivators. Microalgae were grown in an accumulative mode on a Goldberg nutrient environment (Yang *et al.,* 2008). The algae culture was kept at (1) a water temperature of 21–23°C; (2) illumination of 8–10 lx; (3) a photoperiod of 8:16 hours (day: night); (4) stirring (churning) 4–5 times a day. The duration of the experiments was 14 days.

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Since ideal hormone concentrations ranging from 10^{-5} to 10^{-6} µmol have been obtained for other strains (Salama *et al.,* 2014; Gonzalez-Garcinu *et al.,* 2016), for *Ph. tricornutum*, concentrations of salicylic acid (0.4×10^{-5}; 1.0×10^{-5}; 1.9×10^{-5}; 2.8×10^{-5}; 3.8×10^{-5} mol.L^{-1}) and gibberelic acid (0.4×10^{-8}; 0.8×10^{-8}; 1.6×10^{-8}; 3.2×10^{-8}; 3.8×10^{-8} mol.L^{-1}) were tested as a growth stimulant (Hebei Guanlang Biotechnology Co., Ltd, China).

Cultivation was carried out in a monoculture. The initial concentration of *Ph. tricornutum* was 1.96 million cells for salicylic acid, and 1.87 million cells for gibberellic acid evaluation. The increase in algae biomass was determined by the indicator of the optical density, measured at 750 nm. The duration of the experiments was 14 days.

The total carbohydrate content was estimated by the method of acid hydrolysis of suspended algae samples. The formed monosaccharide units pass into furfural derivatives. When added to the L-Tryptophan solution, they form colored complexes that absorb light at a wavelength of 540 nm (Laurens *et al.,* 2012).

Sample preparation for protein determination was carried out according to (Herbert *et al.,* 1971). The protein content was determined by the Lowry protein assay (Lowry *et al.,* 1951).

The number of chlorophylls was isolated by the acetone extraction method from the pre-frozen algae biomass (Carneiro *et al.,* 2019). The quantitative content of chlorophylls was determined spectrophotometrically at wavelengths of 630, 647, 664, and 750 nm. Acetone 90% was used as a control (Aminot & Ray, 2002).

The total lipid content was carried out by a method based on the color reaction of vanillin in an acidic environment with lipids, with the formation of intense coloration. The chromogenic groups are hydroxyl and carbonyl (Johnson *et al.,* 1977).

Values were considered significantly different when $P < 0.05$. All statistical analysis was performed using the Excel and STATISTICA® 7.0 software.

RESULTS AND DISCUSSION

The growth of microalgae in the accumulative culture is characterized by the presence of a piece of the linear growth phase. This piece can have a relatively large length in time, and the density of culture sometimes increases tenfold. This growth is associated with a constant rate of biomass production.

We have evaluated the growth in the accumulative culture of *Ph. tricornutum* when introducing various concentrations of phytohormones into the culture environment. The estimation of the biomass growth was carried out by the indicator of the optical density, measured at 750 nm.

From the data of the Figure 1, it can be seen that gibberellic acid in concentrations of 0.39–3.8 $\times 10^{-8}$ mol has a stimulating effect on the growth of cells in culture. The greatest effect of stimulating the growth of the number of cells is observed at a minimum phytohormone concentration of 0.39×10^{-8} mol. The cell culture density in this case was 181% higher than in the control. Other concentrations of gibberellic acid also stimulated growth of the microalgae, but the growth of the culture was about 140% compared to the control.

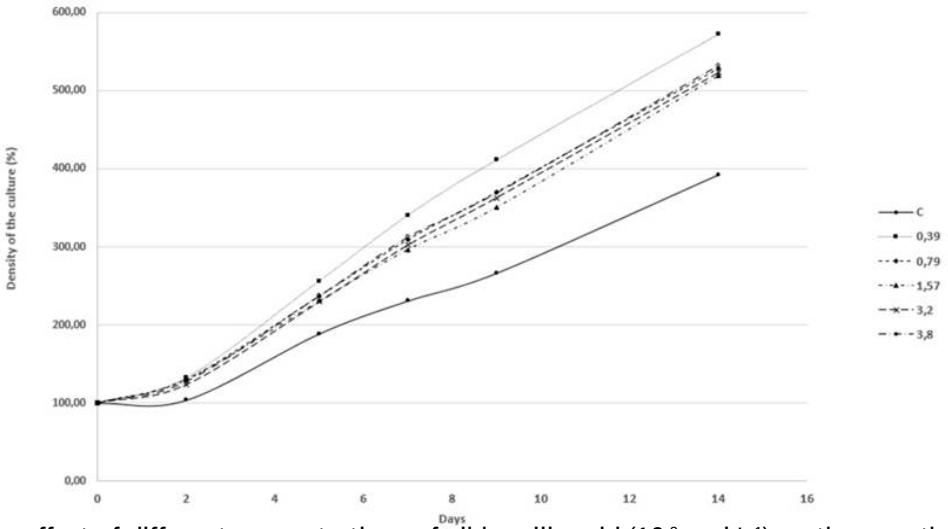

Figure 1. The effect of different concentrations of gibberellic acid (10^{-8} mol.L^{-1}) on the growth of the accumulative culture of *Ph. tricornutum.* The value of the optical density of the culture at the beginning of the experiment was taken as 100%.

Note: C-control; 0.39 – 3.8 -gibberellic acid concentration $\times 10^{-8}$ mol.L^{-1}

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Figure 2 shows the growth dynamics of the *Ph. tricornutum* culture, when various concentrations of salicylic acid are added to the culture environment.

From the data presented in the Figure, it can be seen that salicylic acid is only at a concentration of 0.4×10^{-5} mol.L^{-1} stimulated the growth of *Ph. tricornutum* cell culture. The number of cells on the 14th day of the experiment was 184.6% more than in the control group. Other studied concentrations of phytohormone did not affect the growth of the culture.

In the course of the research, the influence of effective concentrations of phytohormones on the biochemical composition of the *Ph. tricornutum* culture was evaluated.

In the first seven days of the experiment, an increase in the quantitative content of protein in the culture by 28.6%, when using gibberellic acid, was noted, compared with the control (Figure 3).

However, after 14 days of the experiment, the protein content in the experimental groups, using phytohormones, was less than in the control group. It was found that the use of salicylic acid led to a greater accumulation of protein in the culture than when using gibberellic acid. The effectiveness of this phytohormone for protein accumulation did not differ from the control group on the 14th day of cultivation. The effect of gibberellic acid on the 9th–14th day of the experiment was expressed in the inhibition of protein accumulation in the culture, compared with the control group.

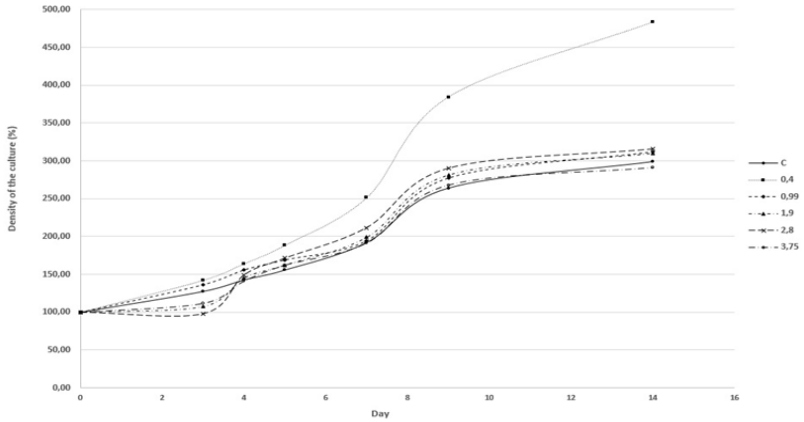

Figure 2. The effect of different concentrations of salicylic acid (10^{-5} mol.L^{-1}) on the growth of the accumulative culture *of Ph. tricornutum.* The value of the optical density of the culture at the beginning of the experiment was taken as 100%

Note: C-control; 0,4 – 3,75 -salicylic acid concentration $\times 10^{-5}$ mol.L^{-1}.

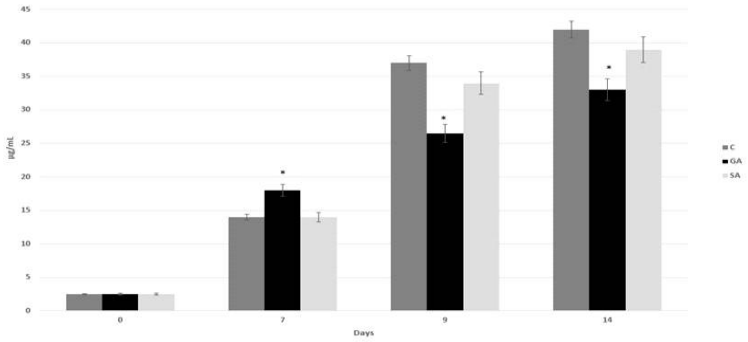

Figure 3. The effect of gibberellic and salicylic acids on the quantitative content of protein in the culture of *Ph. tricornutum.*

Note: C-control; GA-gibberellic acid (0.39×10^{-8} mol.L^{-1}); SA-salicylic acid (0.4×10^{-5} mol.L^{-1}). * — the differences are statistically significant compared to the control ($p \leq 0.05$).

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Evaluation of the carbohydrate content dynamics in the *Ph. tricornutum* culture for seven days revealed the inhibitory effect of phytohormones (Figure 4).

However, on the 9th day of cultivation, higher carbohydrate content was noted in the experimental cultures compared to the control. It should be noted that the carbohydrate content in the control culture and in the culture with the introduction of gibberellic acid on the 14th day of the experiment was equal. However, on the 14th day of the experiment, the carbohydrate content in the culture with salicylic acid significantly decreased, compared to the 9th day of the experiment. The carbohydrate content in this culture was 29.5% less than in the control culture.

The total dynamics of carbohydrate accumulation over the entire period of cultivation in the control culture was 1519.6% (16.2 times), in the culture with gibberellic acid was 1573.9% (16.7 times). The difference in the indicator between these cultures was 3.4%.

Evaluation of the effect of phytohormones on the lipid content, during the entire cultivation period, showed no effect when using gibberellic acid, compared with the control (Figure 5).

At the same time, in the first seven days of cultivation, salicylic acid contributed to the accumulation of lipids by 42.9% more than in the control culture. However, at the end of the experiment, the difference in the lipid content was 18.5%. The overall dynamics of lipid accumulation over 14 days of cultivation revealed significant differences in the increase in their content in the control culture (125%), and in the culture with salicylic acid (166.7%).

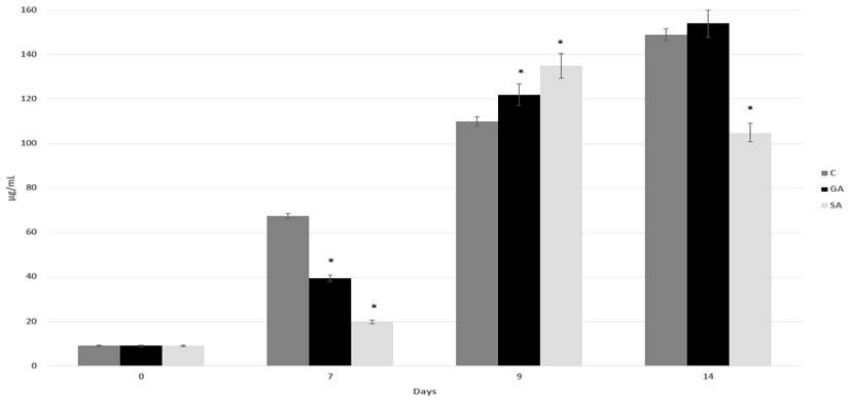

Figure 4. The effect of gibberellic and salicylic acids on the quantitative content of carbohydrates in the culture of *Ph.tricornutum.*

Note: C-control; GA-gibberellic acid (0.39×10^{-8} mol.L^{-1}); SA-salicylic acid (0.4×10^{-5} mol.L^{-1}). * — the differences are statistically significant compared to the control ($p \leq 0.05$).

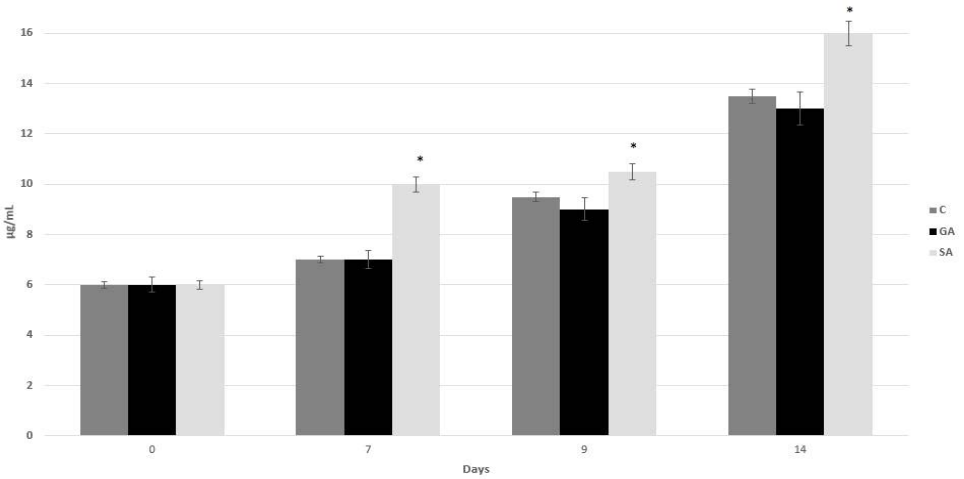

Figure 5. The effect of gibberellic and salicylic acids on the quantitative content of lipids in the culture of *Ph. tricornutum.*

Note: C-control; GA-gibberellic acid (0.39×10^{-8} mol.L^{-1}); SA-salicylic acid (0.4×10^{-5} mol.L^{-1}). * — the differences are statistically significant compared to the control ($p \leq 0.05$).

The research showed that the quantitative content of chlorophyll in experimental cultures, using phytohormones, and in the control culture increased during nine days of cultivation (Figure 6).

Further cultivation did not lead to a significant change in this indicator. We have not detected quantitative differences in the content of chlorophyll when using two phytohormones. The revealed dynamics of the chlorophyll content corresponds to the growth phases of microalgae.

However, optimizing the concentrations of the main elements of the nutrient environment is not the only way to stimulate the growth and to change the component composition of microalgae. It is known that in low concentrations, phytohormones induce cell growth genes, promoting their division (Lu & Xu, 2015). However, in high concentrations, phytohormones can act as herbicides (Tarakhovskaya *et al.,* 2007).

Gibberellic acid in concentrations of $0.79–3.8 \times 10^{-8}$ mol.L^{-1} stimulated the growth of the culture by 127.5%–140.1% more than the control. The maximum effect of stimulation was observed when using a minimum phytohormone concentration of 0.39×10^{-8} mol.L^{-1}. The effect of growth stimulation was 181%. Salicylic acid in low concentrations ($0,4 \times 10^{-5}$ mol.L^{-1}) of 184.6% is characterized by a similar effectiveness of growth stimulation.

The research found that salicylic acid in a concentration of 0.4×10^{-5} mol.L^{-1} stimulated the accumulation of lipids in the *Ph. tricornutum* by 41.7% more than in the control.

The results obtained agree with the data of Xu et al. (2017), which showed that salicylic acid at a concentration of 40 μM with a content of 10 mM KNO_3 in the culture environment stimulated the accumulation of fatty acids (by 28.68%) in the *Ph. tricornutum* culture, compared to the control.

At the same time, gibberellic acid in all the studied concentrations did not stimulate the accumulation of lipids in the *Ph. tricornutum.* Apparently, the investigated concentrations of phytohormone do not have a stimulating effect on lipid accumulation. However, it should be noted that the introduction of 12.4-dichlorophenoxyacetic and abscisic acids into the culture environment, increased the production of biomass and the amount of lipids in *Ph. tricornutum* (Zhang *et al.,* 2021). Gibberellic acid at a concentration of 0.5 mg.L^{-1} stimulated a 3-fold increase in lipid accumulation during cultivation of the green algae *Chlorella pyrenoidosa* (Du *et al.,* 2017).

This research found that salicylic acid stimulated the carbohydrate content only in the first nine days of cultivation. In the culture of *Ph. tricornutum*, treated with salicylic acid, the carbohydrate content is 22.7%, and, treated with gibberellic acid, is 10.9% higher than in the control culture.

The phenomenon of carbohydrate accumulation under the action of salicylic acid can be explained by the ability of microalgae to resist oxidative stress by increasing glycolysis. It is confirmed by an increase in the amount of pyruvate and adenosine triphosphate (ATP). The result of a 14-day experiment showed that the carbohydrate content in the control is 1.5 times higher than with the action of salicylic acid.

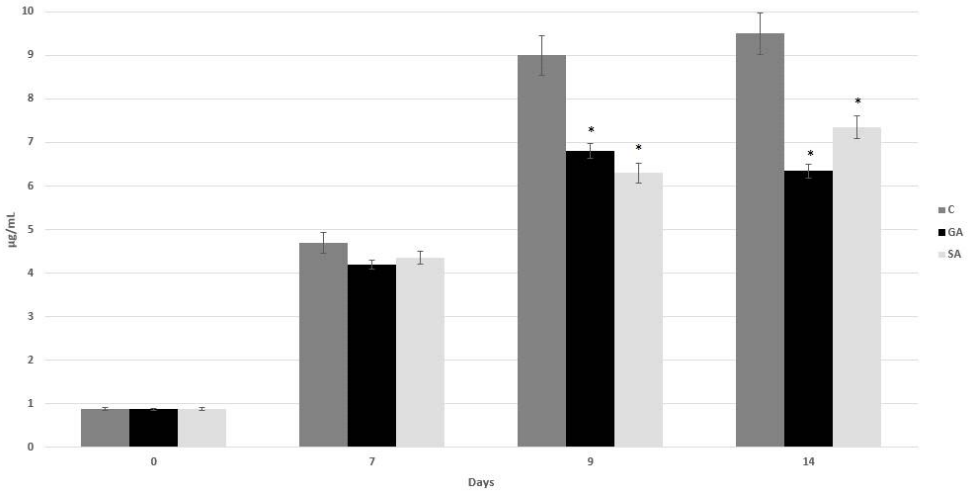

Figure 6. The effect of gibberellic and salicylic acids on the quantitative content of chlorophyll in the culture of *Ph. tricornutum.*

Note: C-control; GA-gibberellic acid (0.39×10^{-8} mol.L^{-1}); SA-salicylic acid (0.4×10^{-5} M). * — the differences are statistically significant compared to the control ($p \leq 0.05$).

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A decrease in the quantitative content of carbohydrates, and an increase in the amount of lipids under the influence of salicylic acid, seems to indicate that short-term oxidative stress of microalgae contributes to a quantitative increase in lipids in them (Bai *et al.,* 2016). Indirect confirmation is the indicators of the content of chlorophyll. In the control culture, the content of chlorophyll increased by 10.2 times, in cultures with the use of phytohormones by 7.2–7.7 times. Further cultivation of the *Ph. tricornutum* culture did not significantly change the indicator in all cultivation variants.

The research showed that each of the phases of microalgae growth is characterized by the direction of carbon metabolism in cells: (1) in the logarithmic phase - protein is synthesized; (2) in the phase of slowing down the growth rate – carbohydrates; (3) in the stationary phase - lipids accumulate. The effect of phytohormones depends on many environmental factors and the phase of plant development – stationary (Xu *et al.,* 2017) or logarithmic (Chu *et al.,* 2019).

Exogenous gibberellins significantly reduce the lag phase and stimulate cell division and growth in the exponential phase of microalgae growth, increase the indicators of total biomass, and promote protein accumulation. Meanwhile, gibberellic acid did not affect the accumulation of lipids and chlorophyll in the culture of *Ph. tricornutum.*

CONCLUSION

The research showed that salicylic and gibberellic acids are effective growth stimulators of microalgae *Ph. tricornutum*. Both phytohormones stimulated carbohydrate accumulation in the microalgae culture and had no effect on protein concentration. The use of salicylic acid significantly increased lipid concentration in the accumulating culture. This type of microalgae is an important component of the diet when cultivating several invertebrates (oysters, scallops, trepang). The use of biotechnological approaches to the production of microalgae biomass using phytohormones is relevant to increase the stability of the food supply of cultivated invertebrates. The revealed features of the biochemical composition of microalgae grown, using various phytohormones, will allow to regulate the intake of nutrients of feed by invertebrates. Also, due to the low cost of phytohormones, their use in the cultivation of microalgae is economically effective.

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