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INFLUENCE OF MUSSEL *MYTILUS GALLOPROVINCIALIS* EXOMETABOLITES ON R-PHYCOERYTHRIN CONCENTRATION IN RED ALGA *GELIDIUM SPINOSUM* WHEN GROWN IN POLYCULTURE

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To increase R-phycoerythrin concentration in red Black Sea alga *Gelidium spinosum* (S. G. Gmelin) P. C. Silva, 1996 (Rhodophyta), it was cultivated in laboratory conditions in polyculture microalga *Tetraselmis viridis* – mussel *Mytilus galloprovincialis* – *Gelidium*; the results of the study are presented. The positive effect of mussel exometabolites on R-phycoerythrin concentration in *Gelidium* in polyculture is described. The relevance of the work is determined by the value of R-phycoerythrin, which is used as a powerful antioxidant, as well as a marker in cytometry and microscopy. The aim of the study is to increase R-phycoerythrin concentration in *Gelidium* using the polyculture method. As a material, *Gelidium* from the fouling of rocks and coastal protection structures of Karantinnaya Bay (Sevastopol) was used; it was cultivated in a laboratory installation with eight working volumes, four of which contained mussels. Mussel decontamination, supplemented with mineral salts and biogens, was used as a nutrient medium for *Gelidium*. The combination of mussel exometabolites with previously developed nutrient medium, based on Black Sea water and enriched with nutrients and mineral salts, results in an increase in R-phycoerythrin concentration by more than 2 times, while the addition of exometabolites to pure filtered seawater increases it maximum by 35 %. Approximate ratios of polyculture elements in 1.5-L volumes, allowing to achieve the desired results in 2 weeks, are as follows: 2 g of *Gelidium* / 50–60 g of two-year-old mussels / 0.4–0.6 g of microalga wet weight.

Keywords: cultivation, polyculture, microalgae, molluscs, macrophytes, nutrient medium

Black Sea *Gelidium spinosum* (S. G. Gmelin) P. C. Silva, 1996 is a valuable raw material source of high-quality agar and R-phycoerythrin – phycobiliprotein pigment, which is widely used in immune diagnostics, microscopy, and cytometry [13]. Algae containing agar and R-phycoerythrin are cultivation objects in many countries of the Asia-Pacific [9 ; 14], and the cost of 1 g of purified R-phycoerythrin reaches $3250–14000 [12].

In previous years, we carried out studies to determine the optimal conditions for *Gelidium* growth and R-phycoerythrin accumulation in it: concentrations of mineral salts and biogens, light and temperature conditions, carbonation of the nutrient medium, as well as its circulation and flow rate [2].

It is known that under natural conditions, *Gelidium* is often found as an epibiont of molluscs-filtrators. Such symbiosis is caused by the positive effect of mollusc exometabolites on macrophyte growth, and that is why hydrobiont cultivation in polyculture has been developed [7 ; 14].

Previously, we obtained results on the beneficial effect of exometabolites of *Anadara kagoshimensis*, starving in pure Black Sea water for 15 days, on *Gelidium* growth and R-phycoerythrin content. With an increase in *Gelidium* biomass by 11.6 %, R-phycoerythrin concentration increased by 40 % compared with the control [4].

The aim of this work is to increase R-phycoerythrin concentration during *Gelidium* cultivation in polyculture. To achieve the aim, the following task is set: to determine the optimal ratio of polyculture elements microalga – mussel – macrophyte.

MATERIAL AND METHODS

As a material, *Gelidium spinosum* from the fouling of rocks and coastal protection structures in the area of Martynova and Karantinnaya bays (Sevastopol) was used. It was cultivated in a laboratory installation with eight working volumes [1] at a temperature range of +15…+27 °C and illumination of 10–25 klx in the mode of 18 h day : 6 h night. The nutrient medium was prepared on the basis of filtered Black Sea water with an increase in its salinity to 26 ‰ and addition of nitrogen, phosphorus, iron, magnesium, and manganese [3]. Mussels (*Mytilus galloprovincialis* Lamarck, 1819) of 45–50 mm were picked from farm collectors located south of the entrance to the Sevastopol Bay, opposite IBSS radiobiological building. Microalga was cultivated separately in a flat cultivator.

Four of eight working volumes of the installation with a right-angled bottom were modernized to contain mussels: they were blocked by left-angled perforated shelves for placing there 2 to 6 individuals with an average weight of 9.5 to 11.5 g. Constant bubbling of the working volumes with air was regulated so that mollusc feces did not stir up and remained in the deepened bottom part.

Mussels were fed with a suspension of *Tetraselmis viridis* (Rouchijajnen) R. E. Norris, Hori & Chihara, 1980 culture, taken from museum of IBSS biotechnology and phytoresources department, either once or twice a day or once every two days. Culture density was of 12–17 mg of microalga wet weight per 1 ml, ranging 5 to 35 ml per one working volume. The contents of the working volumes with mussels were completely poured into previously dried containers with *Gelidium* once every two days; in these containers, a set of minerals and biogens was added [2]. Measurements of R-phycoerythrin concentration in *Gelidium* were carried out by a standard method [10] once a week and at the end of the experiment. Macrophyte initial weight in each working volume was of (2.00 ± 0.05) g. Measurements were made using Sartorius L 220 S balance.

Experiments were carried out in autumn and winter. In the first experiment, decontamination was poured into the containers with *Gelidium* from the working volumes with mussels (4 ind. in each), maintained in clean filtered seawater with the salinity of 26 ‰ and fed with *Tetraselmis viridis* microalga. Thus, *Gelidium* was fed exclusively with mussel metabolites, without mineralization of the medium, the same as in the experiment with *Anadara* [4].

In the second experiment, the components of a previously developed nutrient medium were added to the poured decontamination: nitrogen (8.54 mg per L as KNO_3), phosphorus (1.77 mg per L as KH_2PO_4), iron (1.39 mg per L as $FeSO_4 \cdot 7H_2O$ in combination with 17 mg of Na_2EDTA per 1 g of salt), manganese (0.55 mg per L as $MnCl_2 \cdot 4H_2O$), and magnesium (120 mg per L as $MgSO_4 \cdot 7H_2O$) [2]. Four mussels with an average weight of 9.5–11.5 g were placed into each of four modernized working volumes, and (2 ± 0.05) g of *Gelidium* were placed into four other volumes. Mussels were fed with microalga: 5 to 20 ml of suspension per day.

The first two experiments were carried out according to our patent [3] with an element of *Gelidium* weekly "maturing" to increase R-phycoerythrin concentration. In the third experiment, this element was excluded because of weekly measuring of pigment concentration.

In the third experiment, the same as in the second one, *Gelidium* was grown on mussel decontamination with the addition of minerals and biogens. To identify the dynamics of pigment accumulation in *Gelidium* and to determine the optimal weight ratio of polyculture elements, different number of mussels were maintained in the containers: 3, 4, and 5 ind. with a total initial weight of 33.4, 41.4, and 57 g. They were fed with *Tetraselmis viridis* suspension in the amount of 15, 25, and 35 ml, respectively. Microalga culture density was of 17 mg of wet weight per 1 ml and was kept constant throughout the experiment. In two control working volumes (No. 1 and 5), *Gelidium* was cultivated on the medium mentioned above [2] without metabolites added; it was supplemented with minerals and biogens only. R-phycoerythrin concentration was determined after two, three, and four weeks of cultivation.

The peculiarity of the third experiment was as follows: *Gelidium* was cultivated in five working volumes (No. 1–5) with equal initial mass of 2 g. In three volumes (No. 2–4), the number of added metabolites increased consistently due to both different number of mussels (3; 4; 5) from the volumes No. 6–8 and increased diet of the molluscs (5; 6.25; 7 ml of culture per ind.).

RESULTS AND DISCUSSION

The results of the first two experiments are presented in Tables 1 and 2, of the third one – in Table 3 and in Fig. 1.

Table 1. R-phycoerythrin concentration in *Gelidium* grown on exometabolites of Black Sea mussels with different microalga diet

No. of the experiment	Volume of *Tetraselmis viridis* culture with the density of 12 mg of wet weight per ml	R-phycoerythrin concentration, mg per g
Control	0	5.8 ± 0.5
1	10	6.4 ± 0.9
2	15	5.6 ± 0.4
3	20	7.9 ± 0.8

Table 1 shows that R-phycoerythrin concentration in *Gelidium* increased within the range 10 to 35 % in the polyculture microalga *Tetraselmis viridis* – mussel *Mytilus galloprovincialis* – macrophyte *Gelidium spinosum*, when being fed with mussel exometabolites (if considering the result of the second experiment an artifact). The last figure shows the results close to that obtained in the experiment with *Anadara* [4].

Table 2 presents the results of the experiment, in which decontamination was completely poured into the working volumes with *Gelidium* from the volumes with mussels and enriched with a set of biogens and microelements. Four variants of mussel diet were tested; the maximum variant (20 ml per volume) contributed to an increase in R-phycoerythrin concentration in *Gelidium* by more than three times.

The results of the dynamics of weight growth and pigment concentration in the third experiment are presented in Table 3, and R-phycoerythrin accumulation – in Fig. 1.

The results of a 4-week experiment show that on the 12th day of cultivation in variants with the addition of metabolites from the working volumes with 3, 4 and 5 mussels, R-phycoerythrin accumulation exceeds the initial control value by 50–100 %, while in the containers with no metabolites added (volumes No. 1 and 5) – only by 16 %.

Table 2. R-phycoerythrin concentration in *Gelidium* grown on mussel exometabolites with the addition of nutrients and mineral salts into the culture medium

No. of the experiment	Volume of *Tetraselmis viridis* culture with the density of 12 mg of wet weight per ml	R-phycoerythrin concentration, mg per g
Control	0	7.9 ± 0.8
1	5	16.1 ± 1.5
2	10	20.3 ± 4.4
3	15	18.3 ± 2.2
4	20	28.8 ± 4.5

Table 3. Dynamics of weight growth and R-phycoerythrin concentration in *Gelidium* thalli at different number of mussels in the polyculture

No. of the volume	Number of mussels	V_{ma}, ml	*Gelidium* weight, g / R-phycoerythrin concentration, mg per g			
			30.01.2019	11.02.2019*	18.02.2019*	25.02.2019*
1; 5	0	0	2.0 / 6.9 ± 2.3	2.90 / 8.0 ± 2.3	3.00 / 9.6 ± 1.6	3.20 / 11.1 ± 1.2
2	3	15	2.0 / 6.9 ± 2.3	3.00 / 11.4 ± 2.4	2.55 / 8.7 ± 0.9	3.00 / 12.3 ± 1.2
3	4	25	2.0 / 6.9 ± 2.3	3.25 / 12.4 ± 1.8	3.10 / 10.6 ± 0.8	3.22 / 13.6 ± 0.9
4	5	35	2.0 / 6.9 ± 2.3	3.05 / 14.4 ± 1.4	2.85 / 14.9 ± 1.7	3.30 / 14.4 ± 0.1

Note: at the dates marked with an asterisk (*), samples were taken to measure R-phycoerythrin, and *Gelidium* weight was returned to W_0 = 2 g; V_{ma} indicates volume of microalga suspension.

At the end of the 3rd week of cultivation, there was a significant decrease in pigment concentration in *Gelidium* from the working volume No. 2, into which the decontamination was poured from the volume with 3 mussels. It is 24 % lower than the previous result and 29 % lower than the final one. Since algae were equally supplemented with minerals and biogens in all the volumes, it could result from a change in the physiological state of at least one of three mussels, which we did not monitor in our experiments.

This result can be considered an artifact, since nothing was found in this volume that distinguishes it from the rest ones. Meanwhile, in our other experiments, there were emissions of mussel sex products (such cases can be the reason for further separate studies of their effect on R-phycoerythrin concentration in *Gelidium*).

According to the results of 4 weeks of cultivation, it was noted, that pigment levels in *Gelidium* from the working volumes No. 2–4 were arranged in increasing order directly proportional to the expected increase in the number of exometabolites from 3, 4, and 5 mussels. If R-phycoerythrin level in *Gelidium*, cultivated by already known method [3] in the volumes No. 1 and 5, is taken as 100 %, then we can conclude that the increases from the use of mussel exometabolites are of 11, 12, and 30 %, respectively.

The maximum R-phycoerythrin content obtained in the second experiment (Table 2) was 2 times higher than the maximum pigment concentration in the third experiment (Table 3). This is explained by the facts that in the latter case, the algae were taken for measurements immediately after the end of the experiment and the element of "maturing", in which the lag of phycoerythrin accumulation in rapidly growing biomass is eliminated in the dark and at a low temperature, was not used [3]. In this case, the specific rate of biomass weight growth was quite high: the biomass doubled in less than 10 days.

The change in R-phycoerythrin concentration in *Gelidium* with a different number of mussel individuals in the polyculture is clearly shown in Fig. 1. After two weeks of cultivation, when the macrophyte was fed with exometabolites of 5 mussels, we observed a 2-fold increase in R-phycoerythrin content.

After four weeks of cultivation, the difference with the control decreased and was approximately of 25 %. Exometabolites of 3 and 4 mussels also caused an increase in pigment concentration after two weeks of cultivation – by 30 and 40 %.

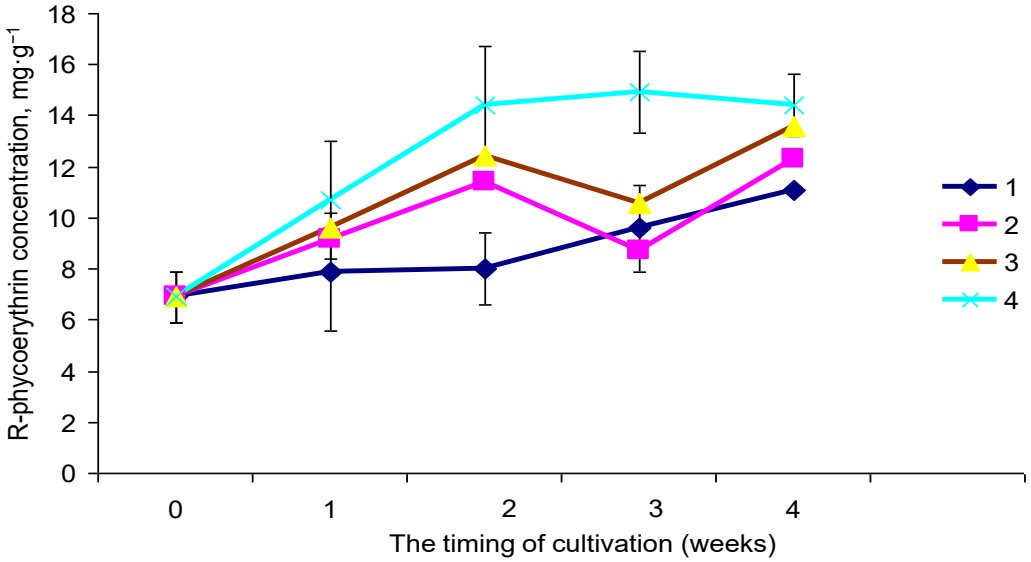

Fig. 1. Dynamics of R-phycoerythrin concentration in *Gelidium* with different number of mussels in the polyculture (1 – control; 2 – 3 mussels; 3 – 4 mussels; 4 – 5 mussels)

The average results of *Gelidium* growth in the volumes No. 2–4 for the last two weeks of the third experiment were of 1 g per week. Thus, a system, including microalga *Tetraselmis viridis* cultivator with a productivity of 600 mg of wet weight per day, a 1.5-L volume for maintaining 5 mussels with a total weight of 60 g, and a 1.5-L *Gelidium* cultivator, is quite capable of producing 14.4 mg of R-phycoerythrin in 7 days.

The advantages of macrophyte cultivation in polyculture in comparison with cultivation in monoculture are known from literature. Thus, macrophytes accumulate larger biomass and larger amount of protein [8 ; 14], and agar quality of *Gracilaria* improves due to creating an optimal diet resulting from exometabolite extraction by invertebrates [7].

Macroalgae can extract from water up to 60 % of nitrogen compounds, including up to 95 % of ammonium [14]. Mussels are known to release ammonium nitrogen into the medium [5]. The chromophore group of pigment (phycobilin) is covalently bound to a water-soluble protein such as globulin [13], the building of which requires nitrogen. In addition, phycobiliproteins are considered to be a “depot” of protein in algae cells. They are destroyed primarily by nitrogen starvation [6 ; 11]. Meanwhile, it is highly possible that not only the addition of nitrogen, but also the form of its compound affect R-phycoerythrin level. However, according to the results of previous studies, culture medium contained a sufficient amount of nitrogen [3]. It seems likely that in that case other interactions have an effect (for example, at the level of hormonal regulation). Thus, the use of metabolites, including in polyculture, creates a fundamentally new way of regulating natural processes [8].

Conclusion. A positive effect of mussel exometabolites on R-phycoerythrin synthesis in the polyculture microalga *Tetraselmis viridis* – mussel *Mytilus galloprovincialis* – macrophyte *Gelidium spinosum* was revealed. The addition of mollusc exometabolites into clean filtered seawater gave an increase in R-phycoerythrin concentration in *Gelidium* by 10–35 %, and the addition of exometabolites in combination

with a standard nutrient medium led to an increase in R-phycoerythrin content by more than 2 times. Approximate weight ratios of polyculture elements in 1.5-L working volumes, allowing to achieve the desired result after two weeks, were as follows: 2 g of *Gelidium* / 50–60 g of two-year-old mussels / 0.4–0.6 g of microalga raw weight.

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REFERENCES

1. Belyaev B. N. Tekhnicheskoe obespechenie kul'tivirovaniya makrofitov. *Rybnoe khozyaistvo Ukrainy*, 2001, no. 5, pp. 21–24. (in Russ.)
2. Belyaev B. N., Beregovaya N. M. The influence of culture medium on the quantity of agar, pigments, and growth of *Gelidium spinosum* from the Black Sea. *Morskoj biologicheskij zhurnal*, 2016, vol. 1, no. 4, pp. 3–11. (in Russ.). https://doi.org/10.21072/mbj.2016.01.4.01
3. Belyaev B. N., Beregovaya N. M. *Sposob kul'tivirovaniya chernomorskoi krasnoi vodorosli Gelidium spinosum (Grev.) Born. et Thur (Rhodophyta).* Patent RU 2691579 C2 MK *C12N 1/12 A01G 33/00*. Zayavlen FGBUN IMBI im. A. O. Kovalevskogo 09.11.2017, no. 2017139062. Opublikovan 14.06.2019. Bul. no. 14. (in Russ.)
4. Borodina A. V., Belyaev B. N., Beregovaya N. M. Vliyanie ekzometabolitov mollyuskov *Anadara kagoshimensis* (Tokunaga, 1906) na makrofity *Gelidium* sp. *Science, technology and life – 2014* : International scientific conference, Czech Republic, Karlovy Vary, 27–28 Dec., 2014. [Karlovy Vary], 2014, pp. 66–71. (in Russ.)
5. Vyalova O. Yu. *Osobennosti energeticheskogo i azotistogo metabolizma nepolovozrelykh midii Mytilus galloprovincialis Lam. v usloviyakh eksperimenta* : avtoref. dis. … kand. biol. nauk : 03.00.17 / NAN Ukrainy, In-t biologii yuzhnykh morei im. A. O. Kovalevskogo. Sevastopol, 2000, 17 p. (in Russ.)
6. Gudvilovich I. N. The influence of cultivation conditions on growth and the phycobiliprotein content in the red microalgae *Porphyridium purpureum*. *Ekologiya morya*, 2010, iss. 81, pp. 28–36. (in Russ.)
7. Evdokimov V. V. *Morfofunktsional'naya otsenka gamet i produktsionnye vozmozhnosti gidrobiontov pri razmnozhenii ikh v mono- i polikul'ture* : avtoref. dis. … d-ra biol. nauk : 03.00.11 ; 03.00.08 / Tikhookeanskii nauchno-issledovatel'skii institut rybnogo khozyaistva i okeanografii. Vladivostok, 1991, 39 p. (in Russ.)
8. Evdokimov V. V., Evdokimov A. V. Vzaimodeistvie gidrobiontov v polikul'ture pri vosproizvodstve v kontroliruemykh usloviyakh. *Izvestiya TINRO*, 2002, vol. 131, pp. 373–380. (in Russ.)
9. Kalugina A. A., Gryuner V. S., Sokolova N. N. Agar iz chernomorskoi vodorosli gelidium. *Rybnoe khozyaistvo*, 1964, no. 4, pp. 68–70. (in Russ.)
10. Krasnovskii A. A. Vydelenie fikoeritrina iz krasnykh vodoroslei, ego spektral'nye i fotokhimicheskie svoistva. *Doklady Akademii nauk SSSR*, 1952, vol. 82, no. 6, pp. 947–950. (in Russ.)
11. Los' S. I. Influence of urea on spectral properties of phycobilin pigments of algae. *Al'gologiya*, 2009, vol. 19, no. 1, pp. 25–33. (in Russ.)
12. Minyuk G. S., Drobetskaya I. V., Chubchikova I. N., Terent'eva N. V. Unicellular algae as renewable biological resource: A review. *Morskoj ekologicheskij zhurnal*, 2008, vol. 7, iss. 2, pp. 5–23. (in Russ.)
13. Stadnichuk I. N. *Fikobiliproteiny.* Moscow : VINITI, 1990, 193 p. (Itogi nauki i tekhniki, ser. biologicheskaya khimiya ; vol. 40). (in Russ.)
14. Titlyanov E. A., Titlyanova T. V. *Morskie rasteniya stran aziatsko-tikhookeanskogo regiona, ikh ispol'zovanie i kul'tivirovanie.* Vladivostok : "Dal'nauka", 2012, 362 p. (in Russ.)]

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Приведены результаты исследований культивирования красной черноморской водоросли *Gelidium spinosum* (S. G. Gmelin) P. C. Silva, 1996 (Rhodophyta) в лабораторных условиях в поликультуре микроводоросль *Tetraselmis viridis* — мидия *Mytilus galloprovincialis* — гелидиум с целью повышения концентрации R-фикоэритрина в последнем. Описано положительное влияние экзометаболитов мидий на концентрацию R-фикоэритрина в гелидиуме в поликультуре. Актуальность работы определяется ценностью фикоэритрина, который используют как мощный антиоксидант, а также как метчик в цитометрии и микроскопии. Цель исследования — увеличить концентрацию R-фикоэритрина в гелидиуме с применением метода поликультуры. В качестве материала использовали гелидиум из обрастания скал и берегоукрепительных сооружений в районе бухты Карантинная (г. Севастополь); его культивировали в лабораторной установке с восемью рабочими объёмами, в четырёх из которых содержали мидий. Деконтат мидий, дополненный минеральными солями и биогенами, использовали как питательную среду для гелидиума. Сочетание экзометаболитов мидий с разработанной ранее питательной средой на основе черноморской воды, обогащённой биогенами и минеральными солями, приводит к увеличению содержания R-фикоэритрина более чем вдвое, в то время как внесение экзометаболитов в чистую профильтрованную черноморскую воду повышает его максимум на 35 %. Ориентировочные весовые соотношения элементов поликультуры в 1,5-литровых объёмах, позволяющие достичь желаемого результата уже через две недели, — это 2 г гелидиума / 50–60 г двухлетних мидий / 0,4–0,6 г сырого веса микроводорослей.

Ключевые слова: культивирование, поликультура, микроводоросли, моллюски, макрофиты, питательная среда