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THE ROLE OF COLD ADAPTATION IN CRYOPRESERVATION OF *DUNALIELLA SALINA* TEOD. MICROALGAE

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Abstract. The main aim of this work was to investigate the effect of cold exposure on the potential for the synthesis of certain cellular products of industrial interest with the unicellular green alga *D. salina* Teod., and to study of the effect of cold adaptation on the preservation of cells during freeze-warming. It was shown that cold adaptation stimulates the formation of intracellular lipid globules and the synthesis of β-carotene, which increases the safety of cells grown on a depleted medium and frozen at a slow rate (1deg/min). Rapid cooling to -196 °C was lethal for *D. salina* Teod. cells.

Keywords: *microalgae, Dunaliella salina, stress factors, cold adaptation, cryopreservation, glycerol, β-carotene.*

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1. Introduction

When choosing an object of research, we were guided both by practical application and by the presence in the selected object of structural and functional features associated with the formation of natural resistance to adverse environmental factors. *Dunaliella salina* is a unicellular halophilic green microalgae that lives in saline water bodies under natural stress (deficiency or excess of nutrients, high salt content, temperature, etc.). able to synthesize a some substances, including lipids, glycerol, proline, β-carotene and stress proteins (El Baz *et al*., 2002; Jahnke & White 2003; Goyal, 2007; Kaewkannetra *et al*., 2012; Lu *et al*., 2012; Ben-Amotz, 1975; Vo *et al*., 2017; Montazeri-Najafabady *et al*., 2016).

The main characteristic feature of *Dunaliella* is the biosynthesis of glycerol in the chloroplast and partly in the cytosol. The amount of this osmoregulatory substance closely correlates with the concentration of salts in the habitat of algae, i.e. an increase in the amount of salts stimulates glycerol synthesis (Ben-Amotz, 1975; Ben-Amotz & Avron, 1987; Chen & Jiang, 2009). In addition to glycerol, *D. salina* have been shown to produce a large amount of chloroplast β-carotene spaces (Halim & Webley, 2015; Xia *et al*., 2014). Such combinations (stress factor → synthesis) make *D. salina* a promising object that can be used for biotechnological purposes for the production of commercially products (Schilipaulis, 1991; Tafreshi & Shariati, 2009).

Due to the growing popularity of microalgae, the development of effective methods for storing culture and creating banks of strains is relevant. It will not only

provide for the need of science and biotechnology for viable and stable cultures, but also solve the problem of biodiversity preservation.

In the algological practice, a wide range of methods are used to preserve microalgae in a viable state: content in long-term storage liquid media (Marsalek et al., 1998), agar, alginate (Chen *et al.*, 2003), lyophilization, anhydrobiosis, etc. However, when using these methods there is a risk of contamination, as well as loss of the original genetic qualities. Storage in conditions of low-temperature banks successfully copes with these problems. Cryopreservation allows you to keep cells for a long time without losing the original characteristics. However, at the moment there is no general protocol allowing the cryopreservation of *D. salina* microalga cells with high efficiency and reproducibility of the results.

Numerous studies show that the success of plant cell cryoconservation primarily depends on their prior cold tempering, which is capable of triggering adaptive genetically fixed cell response mechanisms to low positive temperatures. However, to date, the role of cold adaptation of microalgae in the process of cryopreservation has not been studied enough (Mortain-Bertrand *et al.*, 1996).

The uniqueness of many halophilic microalgae lies in the fact that when exposed to stress, they are capable of hypersynthesis of glycerol, lipids and β-carotene – "natural" cryoprotective substances.

The aim of this work was to study the effect of cold exposure on the potential of the synthesis of these cellular products by *D. salina* Teod. and the study of the combined effect of the composition of the medium of cultivation and cold adaptation to increase the safety of cells during freeze-warming.

2. Materials and methods

Unicellular halotolerant microalga *D. salina* Teod. obtained from the collection of the Department of Botany, V.N. Karazin Kharkiv National University.

Microalgae were cultivated to the stationary phase of growth at a temperature of 25±2°C without aeration on two nutrient media containing different amounts of microelements: Artari (Ar) (Masyuk, 1973) and Ramaraj (Rm) (Sathasivam & Juntawong, 2013). Biomass accumulation was carried out in 40 ml culture flasks, which were covered with apolytetrafluoroethylene (PTFE) membrane with a pore size of 0.22 μm (TPP, Switzerland) with round-the-clock white fluorescent illumination of 52.84 micromol of photons m^{-2} s^{-1} (3 kLux).

Adaptation (precultivation) to low temperatures was carried out by exposure of samples in the dark at temperature 4°C for 24 hours.

Cell suspension of 1 ml in polypropylene cryogenic vial, 1.8 mL (Nunc, Sigma-Aldrich) was frozen under controlled conditions at a rate of 1deg/min with the UOP-1 programmable freezer (Experimental Unit for Cryobiology and Cryomedicine of National Academy of Sciences of Ukraine) or by direct immersion in liquid nitrogen (-196°C). The tubes with cell suspensions were warmed in a water bath (30°C) with continuous shaking for 1–2 minutes.

Microscopic studies were performed using an LSM-510 Meta laser scanning microscope (Carl Zeiss, Germany) when excited by a diode laser with a wavelength of 405 nm.

Nile Red (NR) (Sigma-Aldrich) was used as a rapid test to visualize the accumulation of intracellular lipid globules and β-carotene (Halim & Webley, 2015) by

microalgae cells. Preliminarily, NR was dissolved in acetone to obtain a stock solution of the dye. Then, 0.1 mL stock solution NR was added to 1 ml of culture and incubated at room temperature in darkness for 3-5 min.

Autofluorescence and NR fluorescence intensity were recorded using a diode laser with a wavelength of 405 nm and 543 nm.

For stopping the movement of cells during microscopic study, formalin solution was used (5µl per 1 ml of the suspension under study) prepared on an appropriate culture medium.

For an integrated assessment of the preservation of the cells of the microalga *D.salina* Teod., reflecting the state of the cells (size, shape, area, mobility, presence of flagella), the computer program Aim Image Examiner (Carl Zeiss Micro Imaging) was used.

3. Results and discussion

Initially, we studied the effect of freeze-warming on a *D. salina* culture grown on a medium with different salinity and composition. It was assumed that cultivation in a depleted Ar nutrient medium containing sodium chloride (NaCl) 2M (Chernobai & Kadnikova, 2019) would stimulate the intracellular synthesis of glycerol, which may provide a cryoprotective effect during freeze-warming.

A visual comparison of micrographs of intact *D.salina* cells that are in the stationary growth phase, but grown on different nutrient media, revealed a difference in the pigment composition. After cooling-warming of these cell suspensions, it was established that the composition of the nutrient medium and the amount of NaCl affect the safety of the microalgae (Fig. 2, 8).

In Fig. 1 (1) it can be seen that most of the cells frozen to -196°C at a rate of 1 deg/min on medium Rm lost mobility and integrity, whereas the morphometric parameters of cells on medium Ar (Fig. 1 (2)) saved (safety was 49% ± 2%). Rapid cooling to -196°C resulted in 100% cell death, regardless of the type of nutrient medium.

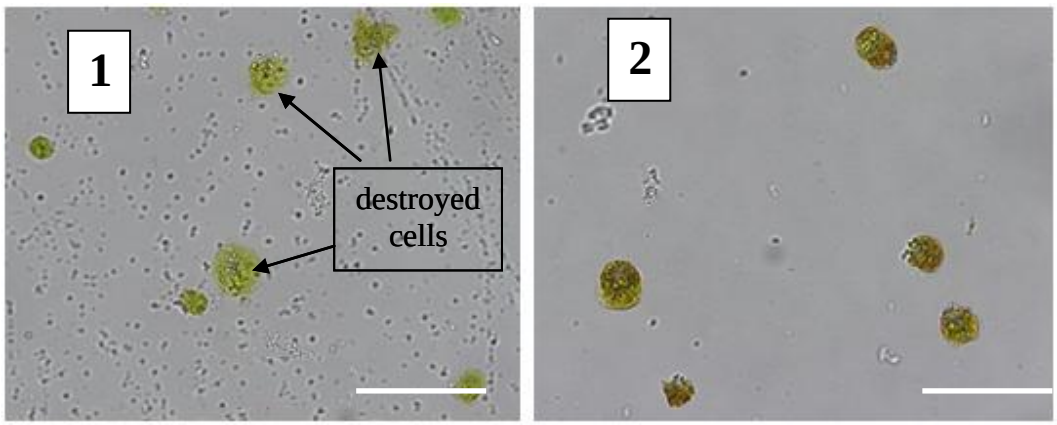


Fig. 1. Light microscopy micrograph of cells after freezing (at a rate of 1 deg/min) and warming: 1 - Rm, 2 – Armedium.Scale bars =50µm

Chlorophyll is known to be sensitive to sudden changes in temperature. Our results confirmed this fact (Fig. 2 (1), 3). After cooling-warming the cells with a rate of

1 deg/min, a significant decrease in the autofluorescence intensity of chlorophyll was observed, while the rapid immersion of the cells in liquid nitrogen had an insignificant effect on this indicator.

The NR fluorescence and the number of lipid globules in the cells was almost the same in the control after the cells cooled at a rate of 1 deg/min (Fig. 2 (2), 3, 7 (2)). As in samples that were frozen by direct immersion in liquid nitrogen, the intensity was so weak that it was not recorded under the experimental conditions. This is due to the fact that with rapid cooling, the integrity of the cells is disturbed, the chloroplast and probably lipid globules disintegrate.

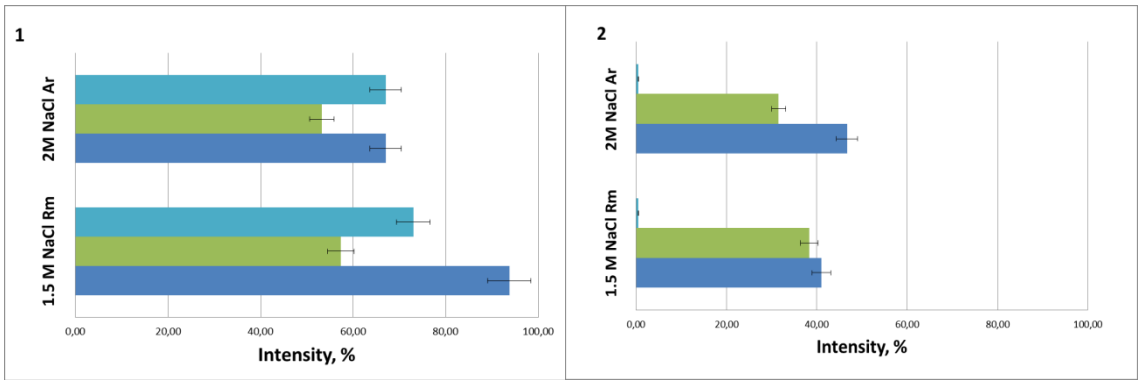
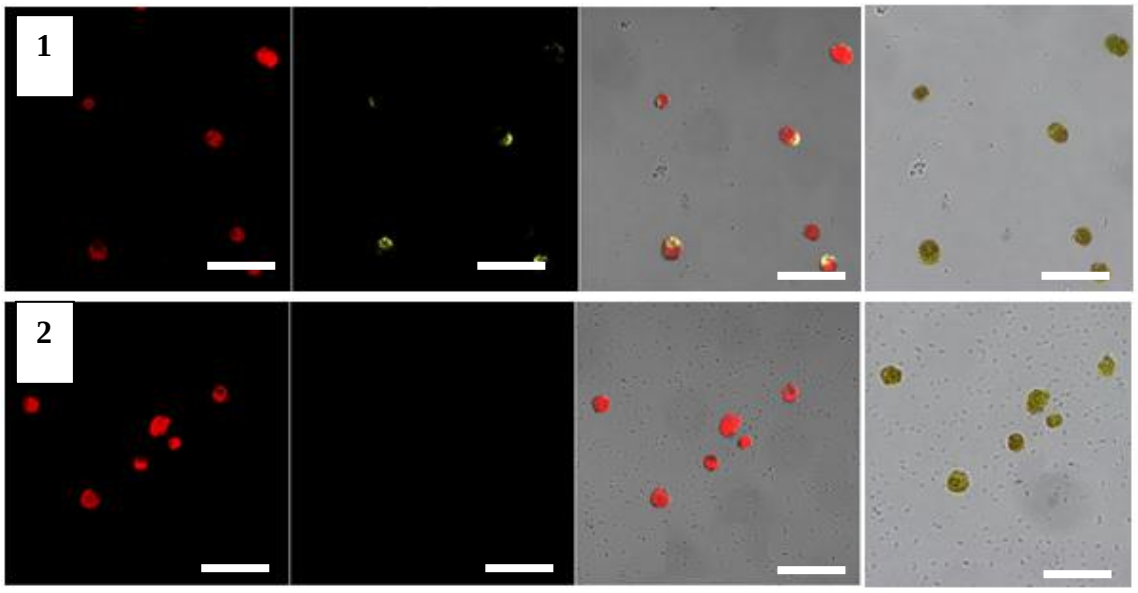


Fig.2. Chlorophyll (1) and NR (2) fluorescence intensity in *D. Salina* microalgae cells depending on the cooling rate and type of nutrient medium:

-freezing by immersion in liquid nitrogen
-freezing at a rate of 1 deg/min
-control

A



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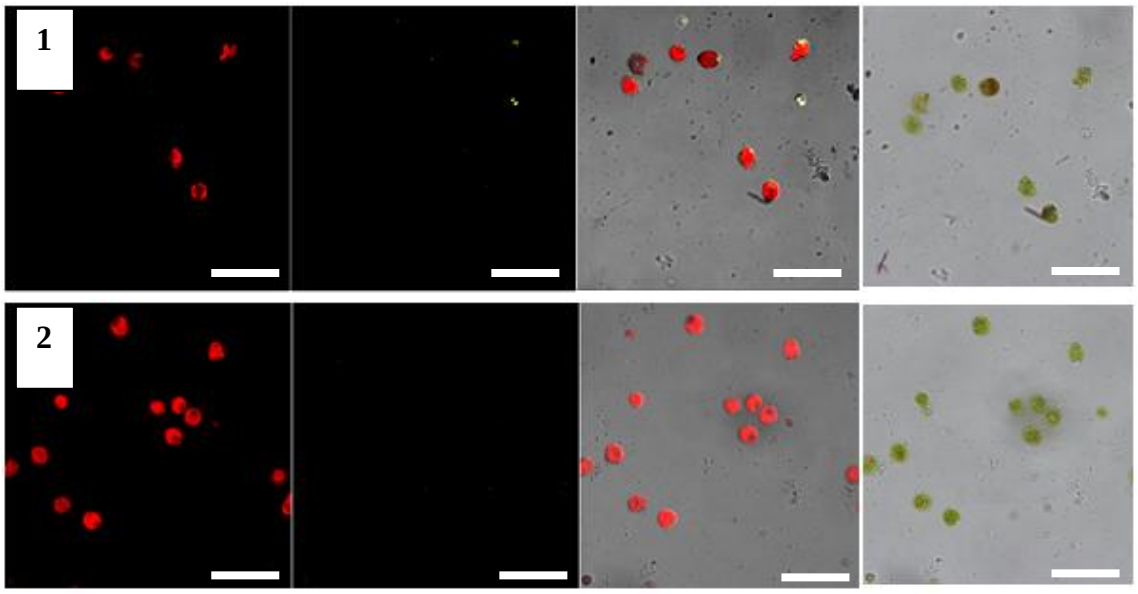


Fig. 3. Fluorescence of chlorophyll (red) and NR (yellow) in *D. salina* cells on medium Ar (A) and Rm (B) after freezing-warming: 1 - with a cooling rate of 1 deg/min, 2 - rapid immersion in liquid nitrogen. Scale bars =50μm

Investigation of the processes of temperature adaptation of *D. salina* cells allows us to assert that under the action of lower temperatures (4°C), the amount of lipid globules containing carotene, their size and the intensity of NR fluorescence in comparison with intact culture is significantly increased. At the same time lipid accumulation, the synthesis of carotene in *D. salina* cultures varied depending on the culture medium. Ar is a depleted nutrient medium with a 2M NaCl concentration. The composition of the medium Rm on the contrary is characterized by a balanced nutrient composition, a ratio of chemical elements and a reduced content of sodium chloride (1.5M) (Fig. 4, 5).

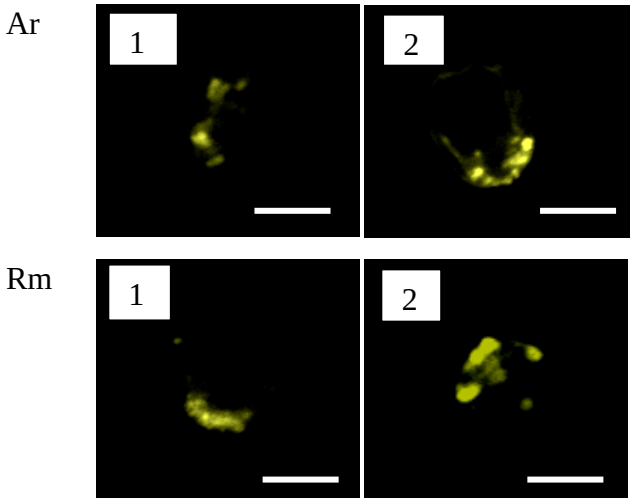


Fig. 4. Microscopic analysis of lipid and carotene accumulation in *D. salina* cultures grown on Ar and Rm media (NR fluorescence, λ =543 nm, scale bars – 10 μm) 1 - control, 2 - after incubation at 4°C for 24 hours

3D-reconstruction of cells *D. salina* (Fig. 5) allowed to establish the localization of lipid globules in which carotenes accumulate. After NR coloring of cells suspension, we were able to establish that the oil globules are at a depth of 7-10 μm with an average cell thickness of 13 μm.

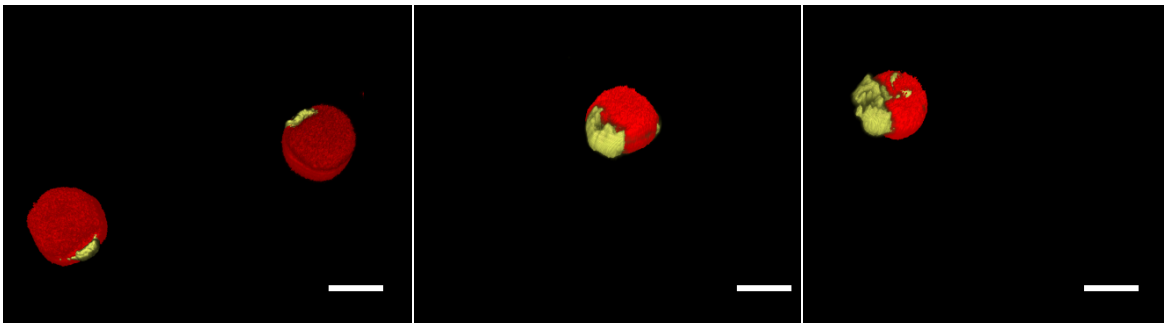


Fig. 5. Localization and number of lipid globules in *D.salina* cells cultured on Ar medium: 1 – control; 2 – pre-cultivation at low temperature for 24 hours; 3 – after freezing at a rate of 1 deg/min. Scale bars =10 μm

It was also found that the freezing-heating of *D. salina* cells at different speeds and after prior adaptation, grown on both nutrient media, resulted in a decrease in the number of lipid globules compared with results obtained after 24 hours of incubation of cells at 4°C (Fig.7, (2)), and, as a result, a decrease in the fluorescence intensity NR, regardless of the cooling rate (Fig. 8).

A

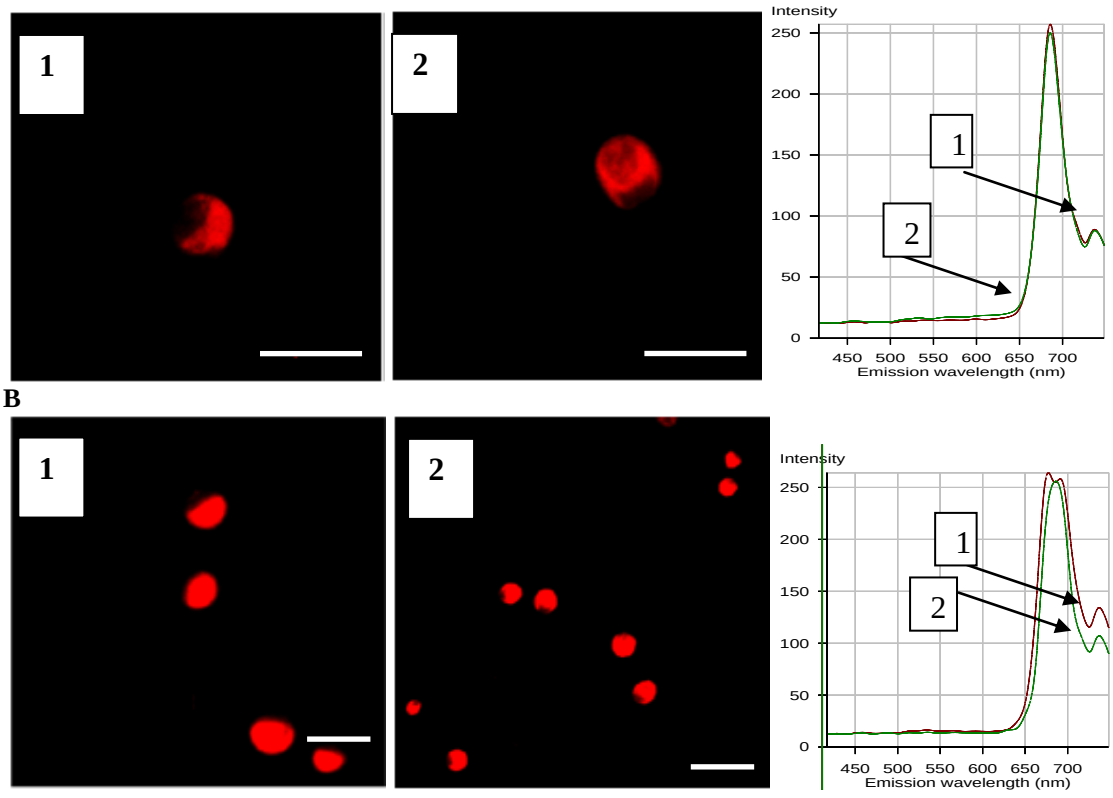


Fig. 6. Fluorescence of chlorophyll (red) in *D.salina* cells on medium Ar (A) and Rm (B): 1 - control, 2 - adaptation at 4°C for 24 hours. Scale bars = 20 μm

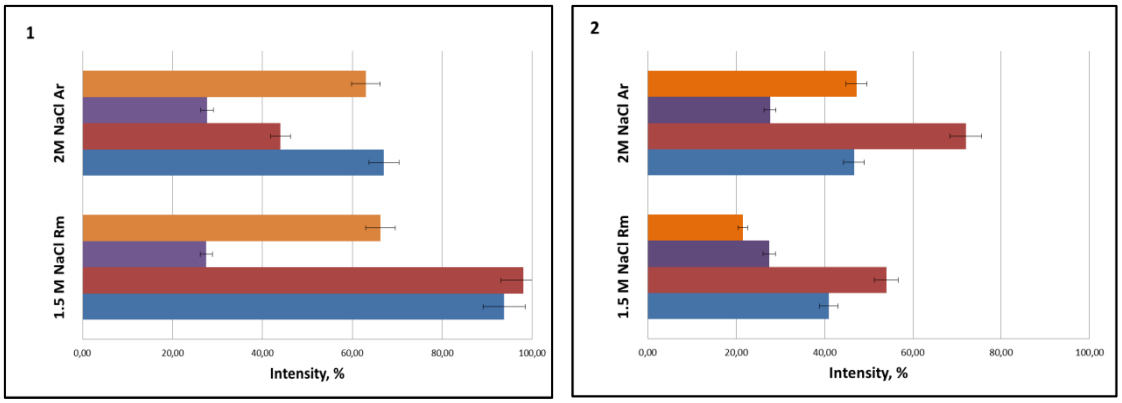
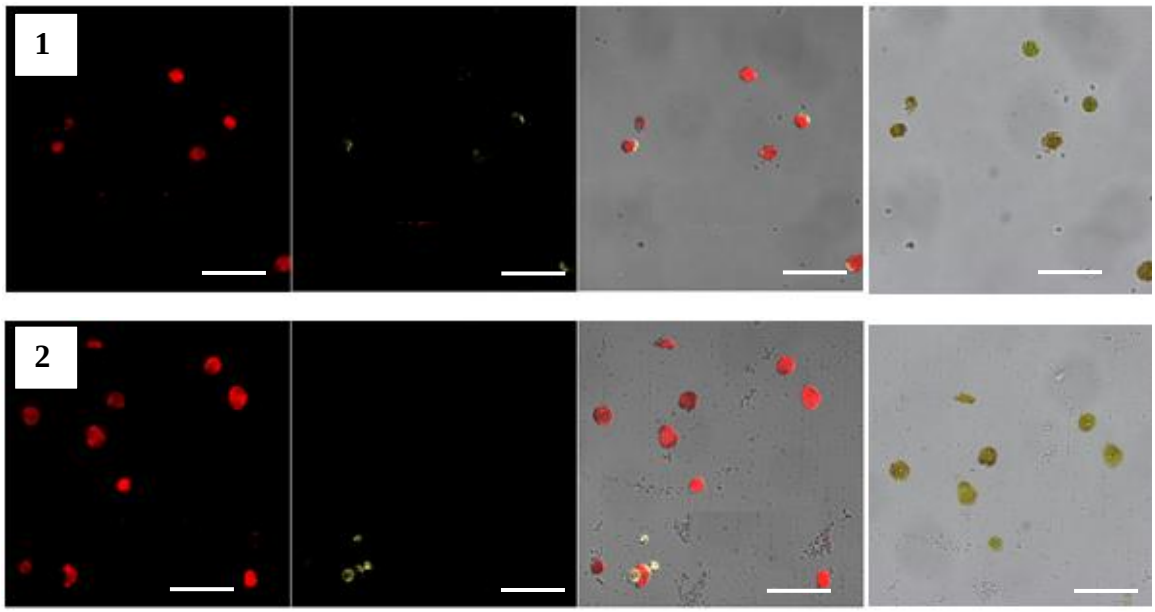


Fig.7. The fluorescence intensity of chlorophyll (1) and NR (2) in the cells of *D. salina* microalgae, depending on the cooling rate and the type of nutrient medium after the preliminary cold adaptation:
-freezing by immersion in liquid nitrogen
-freezing at a rate of 1 deg/min
-incubation at 4°C for 24 hours
-control

A



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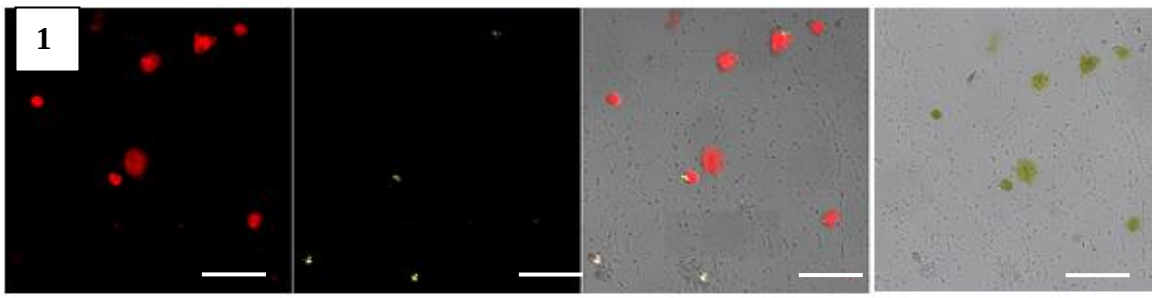

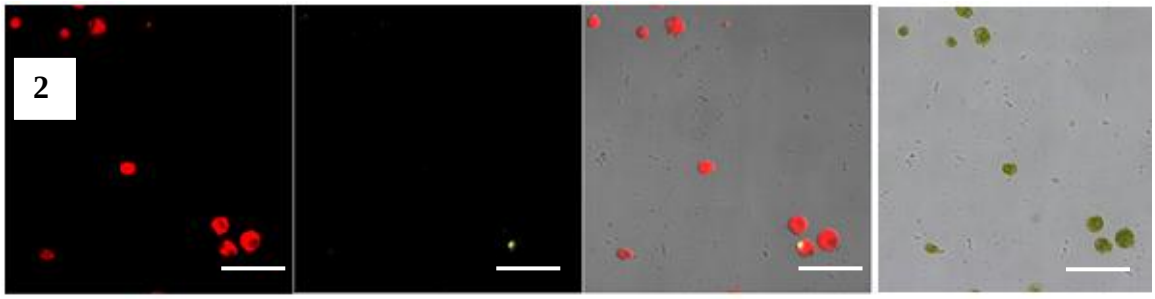

Fig. 8. Fluorescence of chlorophyll (red) and NR (yellow) in *D. salina* cells on Ar (A) and Rm (B) medium with preliminary adaptation after freezing-heating:1 - with a speed of 1 deg/min., 2 - fast immersion in liquid nitrogen. Scale bars =50μm

4. Conclusion

Thus, the preconditions of growth and cold adaptation stimulate the formation of intracellular lipid globules and the synthesis of β-carotene *D. salina* Teod., which in turn increases the safety of cells grown in a depleted medium and frozen at a slow rate (1 deg/min). Rapid cooling to -196°C was lethal for microalgae cells.

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