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The Freshwater Alga Chlorella Sorokiniana Tolerates Salt Stress via Modulating Metabolites and Minerals

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Abstract

Salinity stress is a laborious environmental stress for freshwater microalgae that can be overcame via modulating some physiological traits. This study assessed the impact of salt stress on growth and physiology of the freshwater alga *Chlorella sorokiniana*. The alga was exposed to salt concentrations of 0, 140, 270 and 340 mM NaCl in the BG11 basic growth medium with pH of 7.5 ± 0.20 for 43 d. Growth, in terms of the cell number, of *Chlorella sorokiniana* was progressively decreased with increasing salinity levels. Nevertheless, the salinity-induced reduction in the content of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) was most evident at 270 mM NaCl. Salinity led to progressive increase in the concentrations of protein, soluble sugars, starch and minerals (Na^+ and K^+) and lipids above the control. The present findings suggest that *C. sorokiniana* can withstand salinity stress up to 340 mM via enhancing production of lipids and compatible organic solutes such as proline with minor contribution of protein, soluble sugars and mineral ions (Na^+ and K^+).

Keywords: Biochemical composition; Freshwater Chlorella; NaCl; Salt tolerance.

Introduction

The slow water flow in wetlands and rivers can lead to accumulation of salts which forces algae to manage with these salty conditions either by tolerance or avoidance. The freshwater ecosystems in Egypt are currently experiencing an increasing salinity due to the rising levels of soluble salts in the groundwater and the changes in water flow patterns, leading to the buildup of salt (known as secondary salinization). Secondary salinization leads to environmental alterations, which ultimately impacts the ecosystem particularly the algae (**Nielsen et al., 2003**).

Microalgae can withstand temporary salt stress by adopting mechanisms such as manipulation of the K^+/Na^+ pump to ensure good K^+ selectivity in the cytoplasm in addition to the production of compatible osmolytes like glycerol, glycine betaine, proline, and sucrose (**von Alvensleben et al., 2013)**. These processes help maintain cell turgor and control volume, safeguarding and repairing damaged

proteins, nucleic acids, and membrane lipids (**Erdmann and Hagemann, 2001)**. However, dealing with salinity stress can often reduce biomass productivity due to the energy-intensive nature of osmoregulation (**Oren, 1999**). Additionally, salinity stress is commonly associated with excessive generation of reactive oxygen species (ROS) (**Sudhir and Murthy, 2004**). Interestingly, the natural response of microalgae to salinity stress can modify their biochemical composition. For instance, increased salinity levels can elevate the fatty acid content of freshwater microalgae like *Chlamydomonas mexicana* (**Salama et al., 2014**). The salinity-induced changes in biochemical composition can lead to the swift accumulation of triacylglycerols (TAG) (**Olofsson et al., 2014**) or pigments (**Imamoglu et al., 2009**) in various commercially cultivated algal species.

Adaptation of plant cell to high levels of salts involves three main processes: (1) restoration and maintenance of the cell's internal pressure and size, (2) alternation of cell membrane permeability to enhance the intake of potassium ions (K^+) and release of sodium ions (Na^+) and (3) buildup of specific compatible solutes and stress proteins for protection against osmotic stress (**Erdm ann and Hagemann, 2001**). There are 20 different compatible solutes identified in microalgae, and each can provide varying levels of protection against osmotic stress and salinity. These solutes can impact algal growth, potentially reducing it when a considerable amount of energy is spent in osmotic regulation or when nitrogen is primarily used for synthesizing N-based compounds for osmoregulation, such as proline and glycine betaine (**Vanlerberghe and Brown, 1987; Erdmann and Hagemann, 2001**). There is a limited knowledge regarding the effects of increasing salinity on the growth and performance of freshwater microalgae, particularly *Chlorella sorokiniana*. So, this study examines the influence of salinity on the physiological traits and chemical composition of the freshwater microalga, *Chlorella sorokiniana*.

Materials and Methods

Algal material

Chlorella sorokiniana was collected from Alrukabia Canal, Damietta province, Egypt (31˚43′40″ N and 31˚ 56′57″ E) in May 2021. The collected specimen was identified according to **Guiry and Guiry (2021)**. The alga was cultured on BG11 growth medium as described by **Tran et al. (2010)**, and the medium was regularly refreshed to keep the alga in the exponential growth phase.

Growth media and culture conditions

The working BG11 medium contained (mg/L): $NaNO_3$ 1500, $MgSO_4.7H_2O$ 75, K_2HPO_4 40, , Na_2CO_3 20, $CaCl_2.2H_2O$ 36, citric acid 6.1, EDTA (disodium salt) 1, Ferric ammonium citrate 6, and the trace metals (mg/L): H_3BO_3 2.86, $ZnSO_4.7H_2O$ 0.222, $MnCl_2.4H_2O$ 1.81, $CuSO_4.5H_2O$ 0.079, $NaMoO_4.2H_2O$ 0.39, Co $(NO_3)_2.6H_2O$ 0.0494, the pH was adjusted at 7.5 ± 0.2. The axenic algal culture was incubated under fluorescent light with 30W/m^2 (light/dark cycle 14:10 h) at 25 ± 2°C. The alga was grown on the standard BG11 medium for 7 d before starting the experiments.

Experimental design

Two mL (≈3.8 ×10^6 cells/ mL) of one week-old culture of *C. sorokiniana* were mixed with 200 mL of the sterilized BG11 nutrient medium containing 0, 140, 270, 340 and 514 mM NaCl under aseptic conditions. The algal cultures were incubated under fluorescent light of 30W/ m^2 (light/dark cycle 14:10 h) at 25 ± 2°C. After a growth period 43 d, an aliquot of the algal culture was harvested for estimation of growth and biochemical composition.

Harvest of algal culture

An aliquot (5 ml) of the algal culture was centrifugated at 4000 rpm for 5 min. The pellet was transferred to a clean Eppendorf tube, washed with distilled H_2O, centrifuged twice at 8000 rpm for 5 min and stored at -80°C for biochemical and physiological investigations.

Estimation of algal growth

The number of cells of *C. sorokiniana* culture was assayed by two methods: indirect and direct. The indirect method was conducted by reading the optical density of the cell suspension at 700 nm using a UNICO 7200 series spectrophotometer. By scanning

absorbance of the algal culture across a range of 600–720 nm, the peak of absorbance was found to be 700 nm (Fig. 1). The direct method was conducted by counting the number of cells in the large central square of a Haemocytometer chamber.

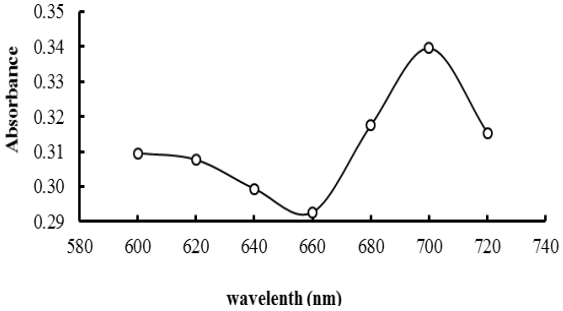

Fig.1 Absorbance of the *Chlorella sorokiniana* culture across a wavelength range of 600–720 nm

Estimation of photosynthetic pigments

The algal photosynthetic pigments were assayed using the procedure of Wellburn and Lichtenthaler (1984). The frozen algal cells were homogenized in liquid nitrogen, and the pigments were extracted in 1ml of 80% acetone (v/v) by grinding in the dark. The homogenate was centrifugated at 8000 rpm for 10 min and the absorbance of the supernatant was read at 470, 645, 662 nm using a UNICO 7200 series spectrophotometer.

The concentrations (µg mL^{-1}) of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoid (Car.) were determined by applying the equations below:

Chl *a* = $11.75 \times A_{662} - 2.35 \times A_{645}$

Chl *b* = $18.16 \times A_{645} - 3.96 \times A_{662}$

Car. = $1000 \times A_{470} - 2.27 \times$ Chl *a* $- 81.4 \times$ Chl *b* /227

Estimation of carbohydrates

A- Total Soluble Sugars (TSS)

Total soluble sugars of the algal pellets were extracted in 1 mL of 90% boiling ethanol in a 1.5-mL Eppendorf. The mixture was centrifugated at 8000 rpm for 5 min and the pellet was kept at – 4 ˚C for determination of starch. The supernatant was heated in an air-forced oven at 60 ˚C until complete evaporation of ethanol and the residue was re-dissolved in 1 mL dist. H_2O and used for estimation of soluble sugars. An aliquot of 100 µL of the extract was completed to 1 mL with distilled water, mixed carefully with 3 mL of the anthrone reagent (8.6 mmol L^{-1} anthrone in 80% v/v H_2SO_4) and heated in water bath at 80 ˚C for 10 minutes, then cooled in an ice bath. The absorbance was measured at 623 nm. The amount of soluble sugars was calculated using a glucose calibration curve in the range 0 to 100 µg glucose per mL (**Schlüter and Crawford, 2001**).

B- Starch

After extraction of soluble sugars, the pellet left was suspended in perchloric acid (9.6 mol L^{-1}), kept in an ice bath for 30 min and centrifugated at 8000 rpm for 5 minutes. The amount of glucose produced from starch hydrolysis was assayed using the anthrone method. Starch content was expressed as glucose equivalents by referring to a glucose calibration curve in the range of 0 to 100 µg glucose mL^{-1} (**Brányiková et al., 2011**).

Estimation of protein

The frozen algal pellet was crushed in liquid nitrogen and mixed with 0.5 ml of 50 mM Hepes buffer (pH 7.4) and 5 µL of 5 mM PMSF. The mixture was thoroughly mixed using a vortex and then centrifuged at 8000 rpm for 5 minutes at 4 ˚C. The amount of protein present in the supernatant was assayed according to Bradford (1976). An aliquot of the extract was made to 1 mL with distilled water followed by addition of 5 mL of the Coomassie brilliant blue reagent, and the absorbance was measured at 595 nm after 5 minutes at room temperature**.** The protein concentration was calculated with reference to a standard curve of bovine serum albumin (BSA) within the 0 to 100 µg per mL.

Estimation of minerals (Na^+ and K^+)

According to **Hansen and Munns (1988)**, Na^+ and K^+ were extracted from the frozen algal pellet in 1mL of boiling distilled water for 2h. The mixture was centrifugated at 8000 rpm for 10 minutes and 1 mL of the supernatant was diluted to 10 mL with distilled H_2O to assay Na^+ and K^+ concentrations by a Jenway PFP7 flame photometer. The concentrations of Na^+ and K^+ was calculated using a standard curve within the range 0–10 ppm Na^+ and K^+.

Estimation of proline

According to **Bates (1973)**, the frozen algal pellet was crushed in liquid nitrogen, then in 3% (w/v) sulfosalicylic acid. The mixture was centrifugated at 8000 rpm for 10 min. One mL of the supernatant was reacted with 2 mL of glacial acetic acid and 2 mL of acid ninhydrin (1.25 g ninhydrin was warmed in 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid with stirring until dissolved) in a water bath at 100 ˚C for 1 h and the reaction was terminated in an ice bath. The red colour was extracted in toluene, the chromophore-containing toluene layer was warmed to room temperature and absorbance was read at 520 nm against toluene as a blank. The concentration of proline was estimated from proline standard curve in a range of 0–50 µg mL^{-1}.

Estimation of lipids

According to **Mishra et al. (2014),** 100 µL dist. H_2O followed by 2 mL concentrated (98%) sulfuric acid were added to the algal precipitate and the mixture was boiled for 10 min and then cooled in an ice bath for 5 min. To the mixture, 5 mL of freshly prepared phospho-vanillin reagent were added. The mixture was incubated in an incubator shaker at 37 °C for 15 minutes at 200 rpm and absorbance was read at 530 nm. The phospho-vanillin reagent was prepared fresh by dissolving 0.6 g of vanillin in 10 mL absolute ethanol and 90 mL deionized water, stirred continuously, then 400 mL of concentrated phosphoric acid was added to the mixture. The reagent was kept in a dark bottle. A standard lipid solution was prepared by mixing 20 mg of commercial canola oil with 10 mL chloroform, this mixture was then stored at a temperature of -20 °C until needed. Aliquots of the standard oil solution were evaporated at 60 °C for 10 minutes to remove the solvent, followed by the addition of 100 µL water. Glass vials coated with Teflon were utilized in all experiments.

Statistical analysis

The data were analyzed using one-way ANOVA with SPSS version 22. For each variable, three replicates were used except otherwise stated. The data were expressed as mean ± SE

Results

Algal growth: cell number versus optical density

The time course of algal growth exhibited a negligible lag phase followed by a long exponential phase (Fig. 2). Algal growth of non-treated cells was assessed by monitoring the turbidity of the culture in terms of absorbance at 700 nm at 2 d intervals. The absorbance at 700 nm (A_{700}) showed a strong positive curvilinear correlation with the cell number estimated directly by the haemocytometer which justifies manipulation of A_{700} to estimate cell number with satisfactory accuracy (Fig. 3).

Effect of salinity on algal physiology

The increase in salinity from 0 up to 340 mM NaCl caused a progressive reduction of 63% in growth of *C. sorokiniana* estimated in terms of cell number (Fig. 4), and algal growth was completely dead at 514 mM NaCl.

The pigment content of *C. sorokiniana* cells decreased progressively by increasing salt level up to 270 mM NaCl, followed by moderate increase at 340 mM NaCl. The magnitude of decrease in Chl a, Chl b and carotenoids across the range 0–270 mM NaCl averaged around 37% (Fig. 5).

The soluble sugars, starch and protein contents of *C. sorokiniana* increased progressively by increasing salt levels from 0 to 340 mM NaCl, and the magnitude of increase amounted to 100%, 90% and 130%, respectively (Fig. 6).

Increasing salinity from 0 to 340 mM NaCl increased Na^+ and K^+ concentrations of *C. sorokiniana* by 1.8 folds and 1.5 folds, respectively, with almost no effect on the K/Na ratio which was averaged around 0.70 at the different salinity treatments (Fig. 7); meanwhile the increase in proline concentration was sharp and amounted to 14.4 folds (Fig. 8) but that in lipid content was mild (only 21%) (Fig. 9).

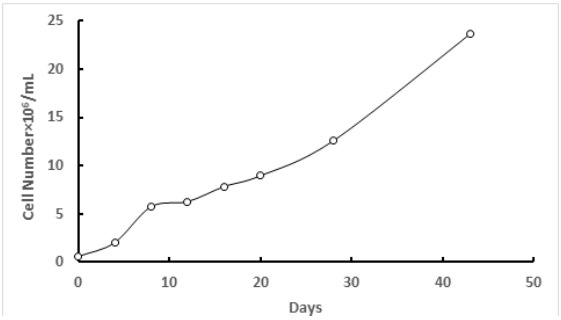

Fig. 2 Growth curve of *C. sorokiniana* on batch culture of the control BG11 nutrient medium

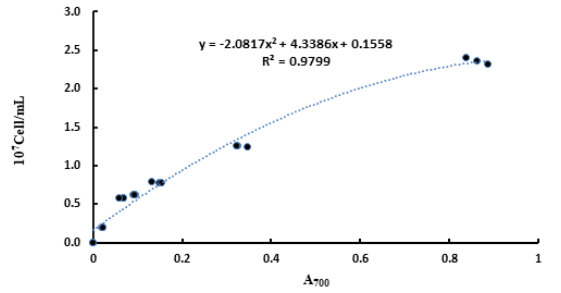

Fig. 3 Relationship between cell number and absorbance of *C. sorokiniana* culture grown on the standard BG11 nutrient medium

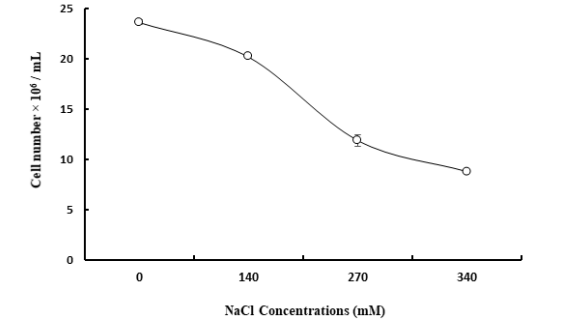

Fig. 4 Effect of NaCl salinity on growth of *C. sorokiniana* estimated as the cell number/mL

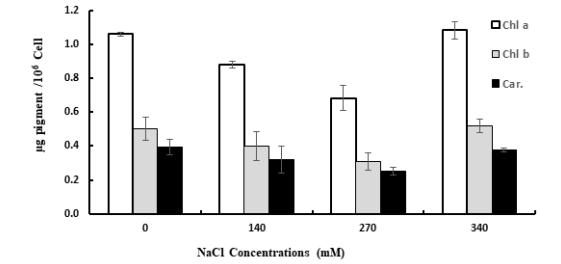

Fig.5 Effect of NaCl salinity on content of photosynthetic pigments of *C. sorokiniana*

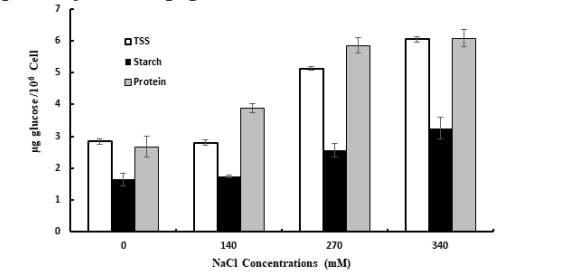

Fig. 6 Effect of salinity on TSS, Starch and protein contents of *C. sorokiniana*

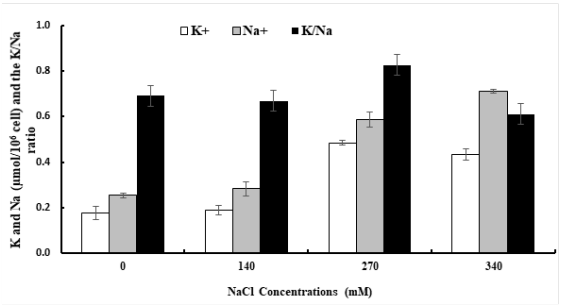

Fig.7 Effect of salinity on Na^+ and K^+ concentrations (µmol Na^+ or K^+ / 10^6 cell) and K^+/Na^+ ratio of *C. sorokiniana*

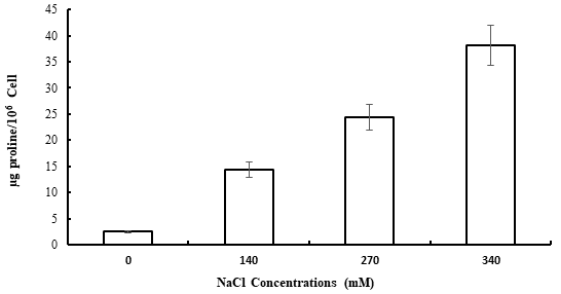

Fig. 8 Effect of salinity on proline concentration (µg proline/ 10^6 cell) of *C. vu sorokiniana*

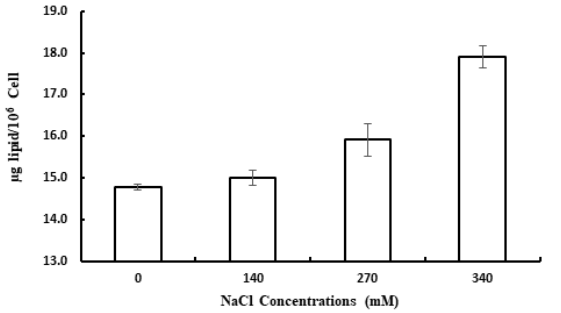

Fig. 9 Effect of salinity on lipid content (µg lipid/ 10^6 cell) of *C. sorokiniana*

Discussion

The intimate positive correlation between cell number and absorbance of the algal culture at wavelength of 700 nm (R= 0.98) justifies the use of culture absorbance as an indirect estimate of cell number and algal growth. In agreement with our findings, **Myers et al. (2013)** adopted the use of culture absorbance as a precise and easy way to estimate algal growth instead of the laborious direct estimation of dry weight, cell number and the level of photosynthetic pigments.

The impact of NaCl on growth and physiology of the freshwater alga *Chlorella sorokiniana* was most pronounced at the highest salt level used (340 mM); yet the impact of NaCl on the content of photosynthetic pigments was most

pronounced at moderate salinity of 270 mM. This points to greater sensitivity of pigment synthesis to salinity stress relative to algal growth and performance. Furthermore, the small rise in pigment content at 340 mM NaCl post the minimum at 270 mM NaCl can attributed to more severe reduction in algal growth than in pigment synthesis by high salinity. Similarly, the increase in photosynthetic pigments in the leaves of drought-stressed *Cynanchum acutum* was related to the drought-induced P deprivation, which can lead to greater reduction in leaf area than in pigment synthesis, rather to drought-induced stimulation of pigment synthesis (**El-Katony et al., 2018**).

The reduction of algal growth, and photosynthetic pigments (Chl *a*, Chl b and carotenoids) under the impact of salt stress may be attributed to the increased uptake of Na^+ and Cl^- leading to ion imbalance, cell dehydration, overproduction of ROS, that is the induction of oxidative stress, enzyme deactivation, and inhibition of photosynthesis activity (Sudhir and Murthy, 2004; Mahajan and Tuteja, 2005; Erdmann and Hagemann, 2001).

The decrease in pigment content by salinity can be accounted for in the light of the claim that salt stress can enhance chlorophyllase activity, thus reducing chlorophyll content (**Mihailovic et al., 1997; Nada et al., 2015**). Interestingly, the increased photosynthetic pigment content by high salinity of 340 mM NaCl, suggests that *C. sorokiniana* can maintain its photosynthetic pigments under high salinity by decreasing activity of chlorophyllase via increasing protein, TSS, starch, minerals, lipid and particularly proline contents. The role of proline in protection of plant cells against salt stress has been documented by **Reynoso and De Gamboa (1982)** who reported that exogenous proline counteracts the impact of high salinity by lowering the buildup of sodium and chlorine ions in *Chlamydomonas reinhardii*. These results suggest that NaCl-treated *Chlorella* has various adaptation pathways to withstand the negative effect of salt stress.

The proline buildup in NaCl-treated *Chlorella* agreed with the findings of **Rasool et al. (2013)** and **Gupta and Huang (2014)** that production of compatible solutes like proline rose to help algae combat the osmotic stress caused by salinity. In other words, an elevated content of free proline is one of the first reactions to various stressful situations (**Mattioli et al., 2009**).

The progressive increase in protein content of *C. sorokiniana* with increasing salinity may be attributed to the fact that proline protects proteins from elevated ion levels by direct engagement with water molecules located on their surface. Increased salt concentration alters the stability of the protein's hydration layer, leading to destabilization of the protein configuration (**Barera and Forlani, 2023**). Proline functions as a compatible osmolyte that helps stabilize subcellular components like proteins and membranes. Additionally, it plays a role in neutralizing harmful free radicals and maintaining the redox balance within cells under the impact of salt stress (**Kishor et al., 2005**). Under high osmotic conditions, proline functions as an osmotrophic molecule by reducing disorder (entropy) and preserving the hydration shell around proteins to keep them stable. The presence of the pyrrolidine ring facilitates bonding with the non-polar sections of proteins, leading to the expansion of their polar region. This adjustment helps offset the adverse impact of high salt concentrations on proteins (**Arakawa and Timashef, 1985**). Also, the amphipathic nature of proline helps membranes to stay stable because it can fit between the different parts of the membrane that are attracted to water (phospholipid head groups) and decreases the damage caused by the oxidation of lipid membranes (**Rudolph et al., 1986**; **Okuma et al., 2004**). Proline can also reduce acid buildup in the cell and keeps the $NADP^+$/NADPH balance at levels suitable for metabolism (**Hare and Cress, 1997**). Furthermore, proline safeguards the protein recycling system from harms caused by stress and increases the production of stress-protecting proteins (**Khedr et al., 2003**).

The present results suggest that *C. sorokiniana* can withstand high salinity through utilizing reducing equivalents to boost the synthesis of proline. This may help decrease the production of reactive oxygen species (ROS) caused by pseudo-cyclic photophosphorylation, thereby preventing potential cellular damage (**Ben Rejeb et al., 2014**). Furthermore, increasing potassium uptake is seen as a beneficial adjustment to cope with high salt levels in salt-tolerant algae. Potassium plays a crucial role in various cellular functions, making it essential to keep the balance between the cytosolic K^+ and Na^+ levels under salt stress (**Shetty et al., 2019**).

Conclusion

The freshwater alga *Chlorella sorokiniana* can tolerate salt stress up to 340 mM NaCl by storing fats as well as proline as an antioxidant, thus protecting the cellular constituents from the negative effects of salt stress. Although increasing NaCl concentration in the culture medium negatively affects cell number and photosynthetic pigments of *Chlorella sorokiniana*, it can lead to enhancement of protein, soluble sugars, starch, minerals (Na^+ and K^+), proline, and lipid accumulation. Therefore, the regulation of water balance using compatible organic compounds along with mineral ions are employed to uphold osmotic balance. The results recommend that the freshwater alga *Chlorella sorokiniana* can tolerate high salt stress and can be cultivated in salt water.

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الملخص العربي

عنوان البحث: : طحلب المياه العذبة Chlorella sorokiniana يمكنه تحمل الإجهاد الملحي عن طريق زيادة نواتج الأيض والمعادن

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يمثل الإجهاد الملحي ظغطا بيئيا شاقا على طحالب المياه العذبة التي تستطيع التغلب عليه بتعديل بعض السمات الفسيولوجية. يهدف البحث الي تقييم تأثير الإجهاد الملحي على النمو والخصائص الفسيولوجية لطحلب المياه العذبة، الكلوريلا سوروكينيانا. تعرض طحلب الكلوريلا لتركيزات ٠، ١٤٠، ٢٧٠، ٣٤٠ مللي مولار من NaCl أثناء نموه في ميديا BG11 ذات الأس الهيدروجيني ٧,٥±٠,٢ لمدة ٤٣ يوم. أوضحت النتائج أن أعداد خلايا الكلوريلا تقل تدريجيا بزيادة درجة الملوحة. ومع ذلك، كان الانخفاض الناتج عن الملوحة في أصباغ التمثيل الضوئي (كلوروفيل أ، كلورفيل ب، الكاروتين) أكثر وضوحا عند تركيز ٢٧٠ مللي مولار من . NaCl ونتج عن تأثير الملوحة زيادة تدريجية في تركيزات البروتين والسكريات الذائبة والنشا والأملاح (مثل الصوديوم والبوتاسيوم (مقارنة بالكنترول. وفسرت النتائج أن طحلب الكلوريلا يستطيع البقاء في ظروف الإجهاد الملحي حتي تركيز ٣٤٠ مللي مولار NaCl عن طريق تحسين انتاج الدهون والبرولين مع مساهمة للبروتين والسكريات الذائبة والمعادن.