Response of Antioxidant Enzymes to Colchicine and Phytohormones Treatments in *Dunaliella salina*

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Abstract

In this experiment, we compared the antioxidative response and biomass production of three ploidy levels induced by three colchicine concentrations (0, 0.1, and 0.5%) and the effects of different concentrations of two phytohormones (auxin and gibberellin) on *D. salina*. The fresh and dry weight of algae treated with colchicine significantly increased. Auxin (1 µM) and gibberellin (10 µM) caused a further increase in biomass in colchicine-treated algae. Colchicine treatment induced catalase and superoxide dismutase activity, but peroxidase activity showed a decrease under this condition. Auxin only increased the superoxide dismutase activity at concentrations of 10 and 100 µM. The catalase activity decreased in the treated algae with 1 and 10 µM auxin. Different auxin concentrations caused an induction in the mentioned enzymes in the colchicine-treated algae. The most increase was observed in catalase activity by adding 1 µM auxin to the colchicine-treated algae. Gibberellin at concentrations of 1 and 10 µM induced peroxidase, catalase, and superoxide dismutase activity. Gibberellin caused a considerable increase in enzyme activity in colchicine-treated algae. The results show that polyploidy along with phytohormones increases the activity of antioxidant enzymes and thus gives the algae the potential for better stress resistance.

Key words: Auxin; Gibberellin; Biomass, Polyploidy; Antioxidant enzyme

Introduction

Dunaliella salina is a green microalga that has many benefits as promising sources for diverse applications, including food for humans, animal feed, and cosmetics due to its provitamin and antioxidant functions. It has been proven that *Dunaliella* is a significant natural source of antioxidants (β-carotene and lutein), to protect against the harsh condition, and glycerol to protect against osmotic pressure (Lamers et al. 2008; Raja et al. 2007).

This alga can be easily cultured in laboratory conditions with a relatively high growth rate and an ability to survive in various environmental conditions compared to other algae. *D. salina* is present at various locations worldwide. The halotolerant

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strains can survive even at 5 M NaCl by maintaining a gradually low intracellular ion concentration, and also by forming compatible solutes such as glycerol; the cells strive to maintain the structure and volume of the cell (Ahmed et al. 2017). This offers an opportunity for commercial biological production of these substances.

Polyploid organisms contain more than two complete sets of chromosomes in the nucleus. Polyploids have naturally induced in different organisms (Baatout 1999). Polyploids can also be experimentally induced in both animals and plants. Colchicine is an alkaloid extracted from seeds or corms of *Colchicum autumnale* L. that can induce polyploidy by tubulin disruption. Polyploid plants may have a wide variety of uses including overcoming hybridization barriers, improving stress tolerance, improving pest resistance, and restoring fertility in wide hybrids (Levin 1983). There are few types of research about the induction of polyploidy in algae and its effects on the physiology and biochemistry of these microorganisms.

It has been proved that phytohormones are present in algae and have regulating roles in metabolism. Phytohormones are widely used as an effective and economical way to achieve high cell density in algal cultures. Gibberellins and auxins are two types of these regulators that involve in numerous aspects of cell growth and development. Positive effects of auxin and gibberellin on the growth and biosynthesis of bioactive compounds have been reported (Lu et al. 2010; Dao et al. 2018; Mansouri and Talebizadeh 2016; Mansouri and Talebizadeh 2017; Mansouri and Nezhad 2020).

In this study, we investigated the effect of colchicine and phytohormone treatments and also their interaction effects on the activity of antioxidant enzymes in *D. salina*.

Materials and methods

Growth conditions

Dunaliella salina was isolated (Sharma et al. 2012) from the Salt River of Shahdad (30° 24′ 16.164″ N 57° 40′ 57.828″ E) in autumn 2017, in Kerman, Iran, and identified based on physiological and morphological descriptions in the references cited in (Massyuk 1973) and Borowitzka and Siva (Borowitzka and Siva 2007; Joseph and Roe 1955). EC and pH of river water were 33.5 ds cm^{-1} and 7.75 respectively. Algae were cultured on the agar plate. After 2–3 weeks, each colony was transferred to a 20 ml liquid growth medium (Artificial Seawater, ASW) with NaCl added to obtain the required salinity medium at 2 M (pH 7.5) (Raja et al. 2007).

The cultures were incubated in a growth chamber under a 16/8 h light-dark provided by cool white fluorescent lamps at an intensity of 49 μmol photons $m^{-2}s^{-1}$ at 25 ± 2°C and were shaken manually twice a day to ensure uniform illumination of the cells.

Colchicine and phytohormones treatments

Haploid cells of *D. salina* were treated with concentrations of 0.1 and 0.5% colchicine (Sigma-Aldrich) and made in culture solution for 36 h. Centrifugation was used

to separate cells from culture media and pellets were washed completely two times with culture solution to free algae from colchicine traces and then were finally transferred to a sterile fresh medium for 21 days. For phytohormone treatment, the fresh culture mediums containing 0, 1, 10, and 100 µM auxin (indol-3-butyric acid) and gibberellic acid (GA_3) (Merk, Hamburg, Germany) in 3 replications were provided, and inoculated with 5×10^6 cells from stock cultures of every colchicine treatment (0, 0.1 and 0.5%). After 3 weeks, all samples were centrifuged and the pellets obtained were then frozen and stored at -70°C before analysis.

Biomass measurement

Biomass was determined by filtering 20 ml of algal culture through a pre-weighed Whatman GF/C filter. The filter with algae was dried overnight at 60°C in a hot air oven and weighed again to estimate the final dry weight. To obtain fresh weight, the Whatman filter was wetted with culture medium and then weighed. After filtering 20 ml algal culture by vacuum pump, the wetted filter with fresh biomass was weighed again.

Enzyme assay

Enzymes were extracted by grinding 0.15 g of fresh algae in a porcelain mortar containing 1.5 ml phosphate buffer containing 50 mM (pH 7.5) ethylene diamine tetraacetic acid (EDTA), 1 mM phenyl methane sulfonyl fluoride (PMSF) and polyvinylpyrrolidone (PVP) 1%. The extract was centrifuged for 15 min at 4°C at 14,000 g and the supernatant was assayed for enzymatic activity and quantification of protein by the Bradford method (Bradford 1976). All operations were performed at 4 °C. The activity of superoxide dismutase (SOD) (EC 1.15.1.1), catalase (CAT) (EC1.11.1.6), and guaiacol peroxidase (GPX) (EC 1.11.1.7) was determined according to the methods used by Giannopolitis and Ries (Giannopolitis and Ries 1977), Azevedo et al. (Azevedo et al. 1998) and Urbanek et al. (Urbanek et al. 1991), respectively.

Statistical analysis

The experiment was arranged in a completely randomized design with three replicates. SPSS software was used for statistical analysis, and graphs were plotted by Excel software. Means were compared using Duncan's multiple range tests at $P < 0.05$.

Results

Changes in fresh and dry weight in colchicine and phytohormones treatments

Effects of auxin on the fresh and dry weight of treated algae were shown in Fig. 1. Fresh and dry weight of algae treated with 1 and 10 µM auxin significantly increased (15 and 42.8% respectively) in comparison to the control. Also in treated algae with colchicine, the fresh and dry weight increased. In colchicine-treated algae, adding 1 µM auxin improved growth, and the highest fresh and dry weights were obtained in the combined treatment of colchicine (0.1 and 0.5%) and 1 µM auxin with 45.89 and 61% increase in comparison to control.

According to the results, the fresh and dry weights increased with increasing

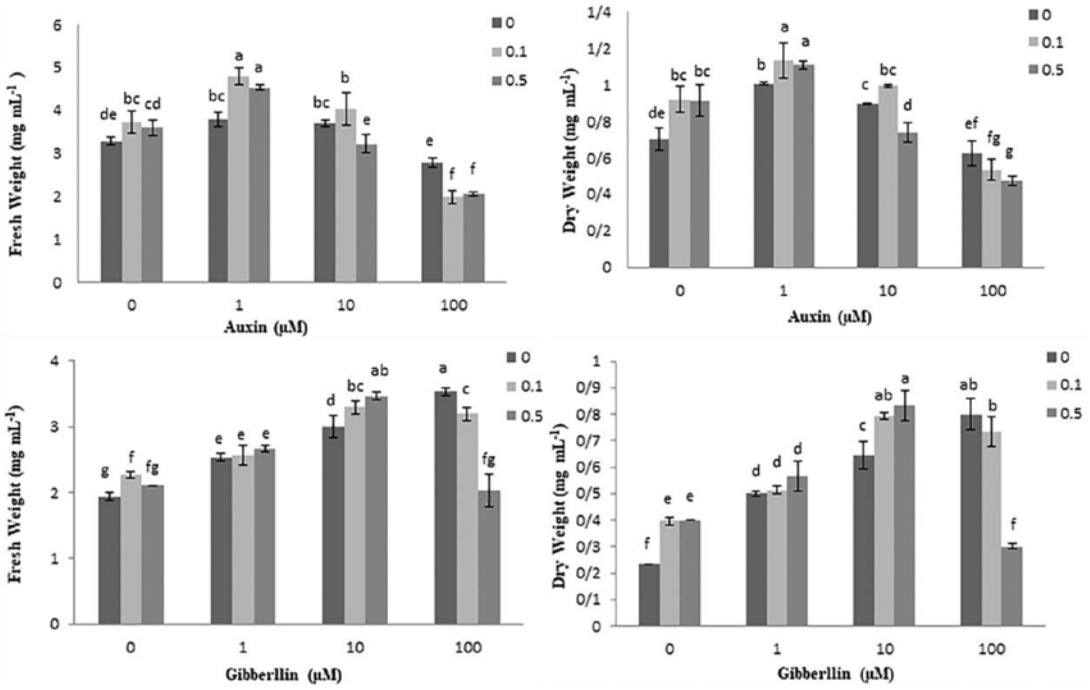

Fig.1. Effect of colchicine (0, 0.1 and 0.5%) and phytohormones (auxin and gibberellin) on fresh and dry weight in *D. salina*. Values are means of three-replications±standard deviation. Different letters show the significant difference with $p < 0.05$ in one-way ANOVA and Duncan's tests

gibberellin concentration to 100 µM (Fig. 1). The enhancement effect of gibberellin was dependent on its concentration. At a concentration of 100 µM Gibberellin, the fresh and dry weights of algae increased by 1.8 and 3.47 times respectively in comparison with the control. Colchicine treatment on both levels significantly increased the fresh and dry weights of algae. The effect of colchicine on dry weight gain was greater than the fresh weight. In algae treated with colchicine, 10 µM gibberellin increased the fresh and dry weights of algae. At 100 µM of gibberellin, fresh weights decreased significantly at both polyploid levels (0.1% and 0.5%). In this regard, 0.5% colchicine had a greater effect on fresh weight loss. A 0.5% colchicine treatment significantly reduced dry weight in treated cultures with 100 µM gibberellin.

Peroxidase activity in colchicine and phytohormones treatments

Treatment with 1 and 10 µM auxin reduced the activity of peroxidase in the *Dunaliella* algae relative to the control (Fig. 2). Both concentrations of colchicine also reduced enzyme activity. The highest peroxidase activity was observed in the treatment of 100 µM auxin and 0.1% colchicine. The 10 µM auxin in the algae treated with both levels of colchicine (0.1 and 0.5%) increased the activity of peroxidase compared to the treatment without colchicine and indicated the effect of polyploidy on increasing the activity of this enzyme.

Results of Figure 2 indicate that 1 and 10 µM gibberellin treatments significantly increased peroxidase activity in comparison

with the control. Colchicine treatment at both levels decreased the enzyme activity. In the simultaneous treatments, the use of 10 and 100 µM gibberellin increased the activity of peroxidase in polyploid algae induced almost 2 times by 0.1 and 0.5% colchicine. The algae treated with both levels of colchicine at a concentration of 1 µM gibberellin showed less enzyme activity than non-polyploidy cultures.

Catalase activity in colchicine and phytohormones treatments

Only 10 µM of auxin increased the catalase activity in comparison with the control (Fig. 3). Algae treated with 0.1% and 0.5% colchicine indicated higher enzyme activity than the control, but there was no significant difference between the two levels of colchicine. Adding 1 µM of auxin to treated algae with 0.1% and 0.5% of colchicine increased the catalase activity by almost 2 times. Furthermore, 100 µM auxin treatment also increased the catalase activity by 34% in algae treated with 0.5% colchicine in comparison to control samples.

Figure 3 shows the results of the effects of gibberellin and colchicine treatments on catalase activity. 1 and 10 µM gibberellin treatments significantly increased the catalase activity. The enzyme showed more

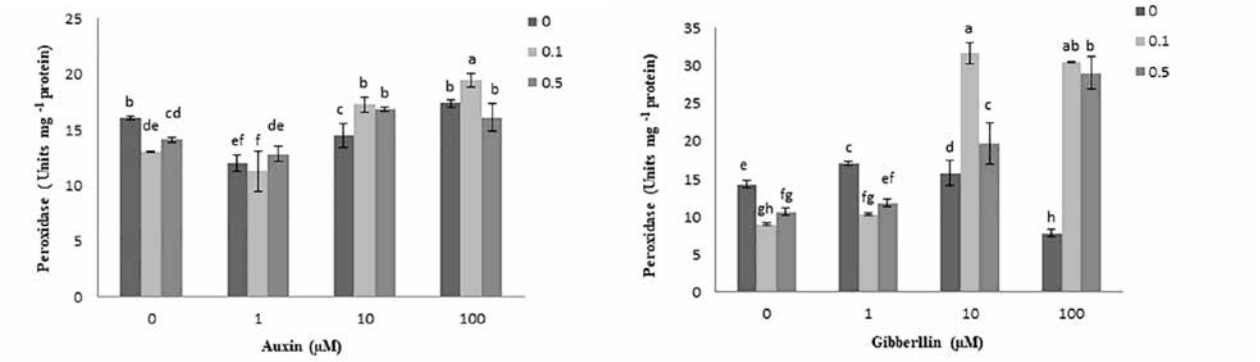

Fig. 2. Effect of colchicine (0, 0.1 and 0.5%) and phytohormones (auxin and gibberellin) on peroxidase activity in *D. salina*. Values are means of three-replications ± standard deviation. Different letters show the significant difference with $p < 0.05$ in one-way ANOVA and Duncan's tests

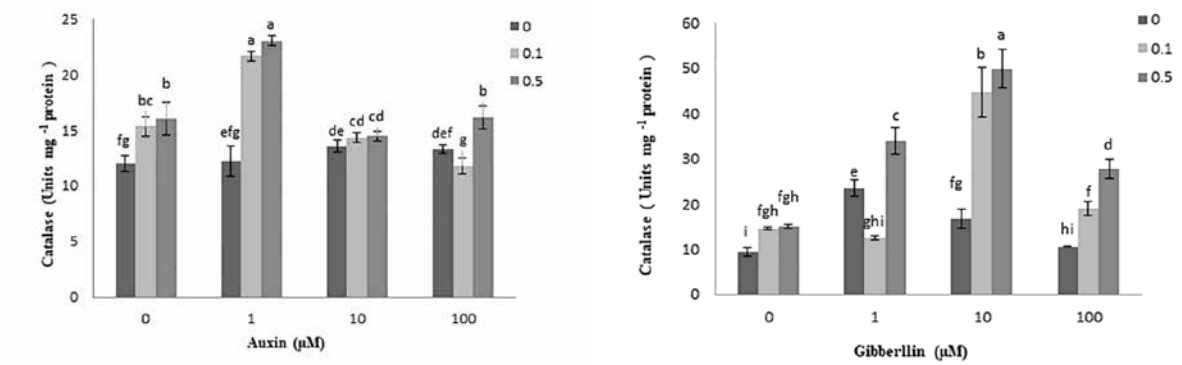

Fig. 3. Effect of colchicine (0, 0.1 and 0.5%) and phytohormones (auxin and gibberellin) on catalase activity in *D. salina*. Values are means of three-replications ± standard deviation. Different letters show the significant difference with $p < 0.05$ in one-way ANOVA and Duncan's tests

activity in algae treated with colchicine. All concentrations of gibberellin (except 1 µM) caused an increase in the catalase activity in colchicine-treated algae. The enzyme activity was 5.3 times higher than the control in the 10 µM gibberellin and colchicine 0.5% treatment.

Superoxide dismutase (SOD) activity in colchicine and phytohormones treatments

The highest activity of superoxide dismutase was seen in the treatment of 100 µM auxin with a 44% increase compared to the control (Fig. 4). The enzyme activity in algae treated with 0.5% colchicine was 14% higher than the control. Auxin with 1 and 10 µM concentrations, significantly increased the superoxide dismutase activity in algae treated with 0.5% and 0.1% colchicine, but samples, which were previously exposed to 0.1% colchicine in the 100 µM auxin treatment, showed less enzyme activity in comparison to control samples.

Gibberellin treatment alone at concentrations of 10 µM and 100 µM significantly increased and decreased the activity of dismutase superoxide (Fig. 4). Colchicine treatment increased enzyme activity only at a level of 0.5%. Adding gibberellin, especially at a concentration of 10 µM, to cultures treated with colchicine significantly increased enzyme activity; the increase in colchicine 0.1% and 0.5% was respectively 3% and 2.7% higher than the control.

Discussion

The present study found the positive effects of all three-treatment groups: auxin, colchicine, and auxin and colchicine on fresh and dry weights of *Dunaliella* algae. The results indicated an increasing effect of auxin on polyploidy cultures. Auxin-induced mitosis significantly increased the number of cells in *Chlorella vulgaris* (Piotrowska-Niczyporuk and Bajguz, 2014). Reports indicated that the biomass of *Chlorella sorokinian* increased in the presence of 1-naphthaleneacetic acid (NAA) compared with the control (Hunt et al., 2010). It has been reported that auxin might increase

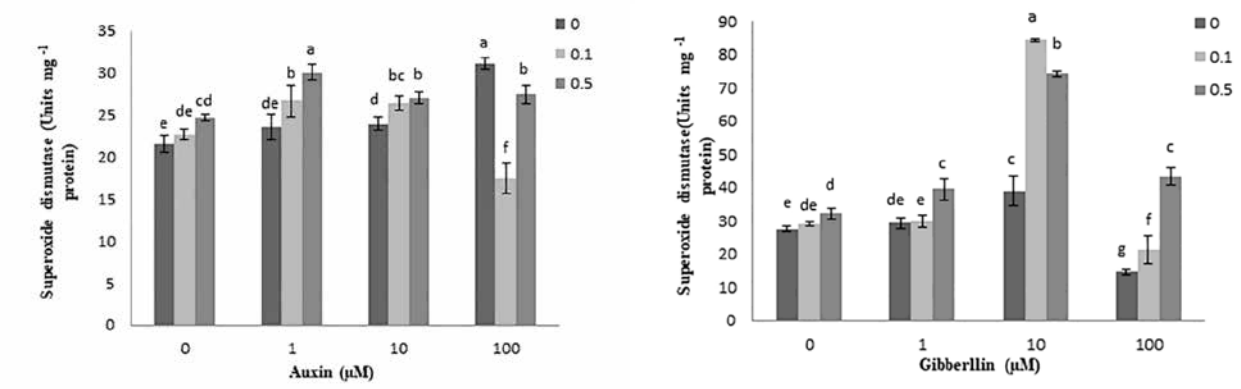

Fig. 4. Effect of colchicine (0, 0.1 and 0.5%) and phytohormones (auxin and gibberellin) on superoxide dismutase activity in *D. salina*. Values are means of three-replications ± standard deviation. Different letters show the significant difference with $p < 0.05$ in one-way ANOVA and Duncan's tests

cell growth through the production of ROS. Some evidence indicates that ROS can be an essential component of the biochemical mechanism involved in loosening during IAA-induced extension growth. It has been indicated that natural auxins (IAA, IBA, and PAA) and synthetic auxins (NAA) decreased the accumulation of ROSs such as H_2O_2 in *C. vulgaris* cells after 48 hours of culture. The finding was according to the antagonistic effects of auxins and ROS on physiological processes. Low levels of ROS have been reported to enhance many cellular processes, including the progression of the cell cycle and the initiation of secondary cell wall differentiation. However, the results indicate that auxin affects algae growth and metabolism through the regulation of ROS levels (Piotrowska-Niczyporuk and Bajguz, 2014). Also, auxin treatment caused an increase in the fresh weight of *Nostoc linckia* algae; and the result was consistent with the results of the present study (Mansouri and Talebizadeh, 2017). As seen in the cultures treated with colchicine 0.5%, the cell division rate decreased in the culture which could be the reason for the reduction of fresh and dry weights of algae (Soltani Nezhad and Mansouri, 2019). Given that the effects of auxin on cell division have been identified, the use of this plant hormone can improve conditions of polyploidy growth. The results of this research indicate the correctness of this hypothesis.

Gibberellins are plant hormones that play important roles in plant growth (Tuna et al., 2008). There are reports of the protective roles of gibberellins in adapting plants to non-biological stresses (Siddiqui et al., 2011). The growth and cell size increase in response to gibberellins have been documented in some algae (Gonai et al., 2004). Results of algae treatment with gibberellin indicated an increase in fresh and dry weights by this hormone so a direct relationship was observed between fresh and dry weights and gibberellin concentration. According to the results of the present study, there was an increase in the number of cells, and thus an increase in the fresh weight of *Chlorella vulgaris* algae treated with gibberellin (Falkowska et al., 2011). These results are similar to those obtained in previous research on blue-green algae, *Nostoc linckia* (Mansouri and Talebizadeh 2016). Also, it was reported that the number of cells and thus dry weight increased in *Microcystis aeruginosa* by GA_3 treatment (Pan et al., 2008).

Treatment with 1 µM auxin reduced the peroxidase activity in Dunaliella algae compared to the control. On the other hand, the highest growth rate was observed in the same treatment. It led to the conclusion that auxin increases growth by reducing oxygen free radicals. In *C. vulgaris*, auxin treatment affected the activity of enzymes involved in ROS scavenging, and levels of H_2O_2 decreased in response to the external application of all synthetic and natural auxins (Piotrowska-Niczyporuk and Bajguz, 2014). In this study, auxin increased the activity of peroxidase in cultures treated with colchicine, indicating the effect of

polyploidy on increasing enzyme activity. The highest activity of superoxide dismutase was seen in the 100 µM auxin treatment, but 1 µM and 10 µM auxin just increased the enzyme activity in polyploid cultures. These results indicated the higher sensitivity of enzymes in polyploidy cells to auxin.

Low concentrations of gibberellin significantly increased catalase and peroxidase activity. Gibberellin treatment increased the catalase and peroxidase activity in all parts of *Catharanthus roseus* in comparison with the control (Jaleel et al., 2010). Gibberellin increased the enzyme activity in cultures treated with colchicine. Treatment with GA3 increased the activity of superoxide dismutase in *C. roseus*. H_2O_2 scavenging systems, which are provided by ascorbate peroxidase and catalase, are more important than superoxide dismutase in coping with oxidative stress (Jaleel et al., 2007). The enzyme activity increased in algae treated with colchicine 0.1% at concentrations of 10 µM and 100 µM gibberellin. The increase was very significant at a concentration of 10 µM.

According to reports, levels of superoxide anhydride and hydrogen peroxide anions were lower in tetraploids than in diploid plants, but the activity of antioxidant enzymes such as superoxide dismutase, peroxidase, catalase, ascorbate peroxidase, and glutathione reductase increased, and antioxidant compounds such as ascorbic acid and glutathione were maintained at high concentrations. Overall results indicated that polyploidy plants had a stronger antioxidant system and were more resistant (Gill and Tuteja, 2010). On the other hand, it is suggested that phytohormones can regulate the synthesis of basic antioxidant enzymes (Szechyńska-Hebda et al., 2007). Auxins may delay protein loss because they stimulate the synthesis of protease inhibitors. In this way, they can increase the number of enzyme molecules and thus increase activity.

Our results showed that treatment with auxin and gibberellin affects the activity of antioxidant enzymes, especially in polyploidy conditions. This feature can give algal cells the ability to cope with stress. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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References

Ahmed RA, He M, Aftab RA, Zheng S, Nagi M, Bakri R, Wang C. (2017). Bioenergy application of *Dunaliella salina* SA 134 grown at various salinity levels for lipid production. Scientific Reports. 7 (1): 1-10.

Azevedo R, Alas R, Smith R, Lea P. (1998).

Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. Physiologia Plantarum. 104 (2): 280-292

Baatout S. (1999). Molecular basis to understand polyploidy. Hematology and cell Therapy. 41 (4): 169-170.

Borowitzka MA and Siva CJ. (2007). The taxonomy of the genus Dunaliella (Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. Journal of Applied Phycology. 19 (5): 567-590.

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72 (1-2): 248-254.

Dao G-H, Wu G-X, Wang X-X, Zhuang L-L, Zhang T-Y, Hu H-Y. (2018). Enhanced growth and fatty acid accumulation of microalgae Scenedesmus sp. LX1 by two types of auxin. Bioresource Technology. 247:561-567.

Falkowska M, Pietryczuk A, Piotrowska A, Bajguz A, Grygoruk A, Czerpak R (2011) The effect of gibberellic acid (GA3) on growth, metal biosorption and metabolism of the green algae *Chlorella vulgaris* (Chlorophyceae) Beijerinck exposed to cadmium and lead stress. Polish Journal of Environmental Studies. 20 (1): 53-59.

Giannopolitis CN and Ries SK. (1977). Superoxide dismutases: I. Occurrence in higher plants Plant Physiology. 59 (2): 309-314.

Gill SS and Tuteja N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry. 48 (12): 909-930.

Gonai T, Kawahara S, Tougou M, Satoh S, Hashiba T, Hirai N, Kawaide H, Kamiya Y, Yoshioka T. (2004). Abscisic acid in the thermoinhibition of lettuce seed germination and enhancement of its catabolism by gibberellin. Journal of Experimental Botany. 55 (394): 111-118.

Hunt RW, Chinnasamy S, Bhatnagar A, Das K. (2010). Effect of biochemical stimulants on biomass productivity and metabolite content of the microalga, *Chlorella sorokiniana*. Applied Biochemistry and Biotechnology. 162 (8): 2400-2414.

Jaleel CA, Gopi R, Manivannan P, Sankar B, Kishorekumar A, Panneerselvam R. (2007). Antioxidant potentials and ajmalicine accumulation in *Catharanthus roseus* after treatment with giberellic acid. Colloids and surfaces B: Biointerfaces. 60 (2): 195-200.

Jaleel CA, Salem MA, Hasanuzzaman M, Nahar K. (2010). Plant growth regulator interactions results enhancement of antioxidant enzymes in *Catharanthus roseus*. Journal of Plant Interactions. 5 (2): 135-145

Joseph H and Roe H. (1955). The

determination of sugar in blood and spinal fluid with anthrone reagent. Journal of Biological Chemistry. 212: 335-343.

Lamers PP, Janssen M, De Vos RC, Bino RJ, Wijffels RH. (2008). Exploring and exploiting carotenoid accumulation in *Dunaliella salina* for cell-factory applications. Trends in Biotechnology. 26 (11): 631-638

Levin DA. (1983). Polyploidy and novelty in flowering plants. The American Naturalist. 122 (1): 1-25.

Lu Y, Jiang P, Liu S, Gan Q, Cui H, Qin S. (2010). Methyl jasmonate-or gibberellins A3-induced astaxanthin accumulation is associated with up-regulation of transcription of β-carotene ketolase genes (bkts) in microalga *Haematococcus pluvialis*. Bioresource Technology. 101 (16): 6468-6474.

Mansouri H and Nezhad SE. (2020). Improvement in biochemical parameters and changes in lipid profile of *Scenedesmus obliquus* by plant growth regulators under mixotrophic condition. Biomass and Bioenergy. 140: 105708.

Mansouri H and Talebizadeh B. (2016). Effect of gibberellic acid on the cyanobacterium *Nostoc linckia*. Journal of Applied Phycology. 28 (4): 2187-2193.

Mansouri H and Talebizadeh R. (2017). Effects of indole-3-butyric acid on growth, pigments and UV-screening compounds in *Nostoc linckia*. Phycological Research. 65 (3): 212-216.

Massyuk N. (1973). Morphology, taxonomy, ecology and geographic distribution of the genus Dunaliella Teod. and prospects for its potential utilization. Kiev, Naukova Dumka Massyuk: 312.

Pan X, Chang F, Kang L, Liu Y, Li G, Li D (2008) Effects of gibberellin A3 on growth and microcystin production in *Microcystis aeruginosa* (cyanophyta). Journal of Plant Physiology. 165 (16): 1691-1697.

Piotrowska-Niczyporuk A and Bajguz A. (2014). The effect of natural and synthetic auxins on the growth, metabolite content and antioxidant response of green alga *Chlorella vulgaris* (Trebouxiophyceae). Plant Growth Regulation. 73 (1): 57-66.

Raja R, Hemaiswarya S, Rengasamy R. (2007). Exploitation of Dunaliella for β-carotene production. Applied Microbiology and Biotechnology 74 (3): 517-523.

Sharma P, Agarwal V, Mohan MK, Kachhwaha S, Kothari S. (2012). Isolation and characterization of Dunaliella species from Sambhar Lake (India) and its phylogenetic position in the genus Dunaliella using 18S rDNA. National Academy Science Letters. 35 (3): 207-213.

Siddiqui MH, Al-Whaibi MH, Basalah MO. (2011). Interactive effect of calcium and gibberellin on nickel tolerance in relation to antioxidant systems in *Triticum aestivum* L. Protoplasma 248 (3): 503-511.

Soltani Nezhad F and Mansouri H. (2019). Induction of polyploidy by colchicine

on the green algae *Dunaliella salina*. Russian Journal of Marine Biology. 45 (2): 106-112

Szechyńska-Hebda M, Skrzypek E, Dąbrowska G, Biesaga-Kościelniak J, Filek M, Wędzony M. (2007). The role of oxidative stress induced by growth regulators in the regeneration process of wheat. Acta Physiologiae Plantarum. 29 (4): 327-337.

Tuna AL, Kaya C, Dikilitas M, Higgs D. (2008). The combined effects of gibberellic acid and salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status in maize plants. Environmental and Experimental Botany. 62 (1): 1-9.

Urbanek H, Kuzniak-Gebarowska E, Herka K. (1991). Elicitation of defence responses in bean leaves by *Botrytis cinerea* polygalacturonase. Acta Physiologiae Plantarum. 13: 43-50.