RESEARCH ARTICLE

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THE AMELIORATIVE EFFECT OF BENZYLADENINE ON THE FRESHWATER MICROALGA CHLORELLA VULGARIS GROWN UNDER NACl STRESS

ABSTRACT:
The present study was undertaken to investigate the influence of benzyladenine (BA) on growth and metabolism of the freshwater microalga Chlorella vulgaris cultures subjected to sodium chloride stress. The interactive effects of salinity (2, 5, and 15 ppt NaCl) and/or benzyladenine (BA, 10 ppm) on growth, pigments composition, carbohydrate constituents, protein content, amino acids concentrations, as well as the endogenous phytohormones levels of the fresh water microalga Chlorella vulgaris were investigated. Cell density, specific growth rate, biomass yield and pigments composition values were decreased with increasing salinization level, except for 2 ppt NaCl which significantly decreased the previously mentioned parameters. Metabolic constituents (carbohydrate fractions, protein contents and amino acids constituents also had the same pattern of response. Increasing salinity decreased the endogenous levels of the growth promoters (Auxin "IAA", "Gibberellic acid" GA₃ and cytokinins) and a significant increase in the growth inhibitor "abscisic acid" (ABA) was attained. Addition of BA improved the inhibitory effects of salinity and osmoregulate cell metabolism either by producing high contents of the low molecular weight molecules ("LMWM" amino acids, protein and soluble sugars) or enhancing growth promoting substances.

KEY WORDS:
Microalgae, salinity, osmoregulation, phytohormones, benzyladenine, metabolism.

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INTRODUCTION:
Unicellular eukaryotic microalgae have a special importance due to the simplicity of their structure, showing all of the metabolic activities and having similarities to the higher plants (Richmond, 1986). Because they lack differentiated tissues and inter tissue transport mechanisms, eukaryotic microalgae may indeed represent a simplified system to elucidate the plant cell response to stress conditions (Tartari and Forlani, 2008).

Fujita et al. (2006) reviewed the abiotic stress that leads to a morphological, physiological biochemical and molecular changes. Drought and/or salinization are manifested as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cells. Oxidative stress, which frequently accompanies high temperature, salinity, or drought stress, may cause denaturation of functional and structural proteins. As a consequence, these diverse environmental stresses often activate similar cell signaling pathways and cellular responses, such as the production of stress proteins, up regulation of antioxidants and accumulation of compatible solutes (sugars, quaternary amines and amino acids). Biochemical adaptation in plants involves various changes in the biochemistry of the cell. These changes include the evolution of new metabolic pathways, the accumulation of low molecular weight metabolites, the synthesis of special proteins, detoxification mechanisms and changes in phytohormone levels. Adaptation represents the ability of a living organism to fit into a changing environment, at the same time improving its chances of survival and reproducing itself (Tartari and Forlani, 2008).

Plant hormones are active members of the signal cascade involved in the induction of plant stress responses (Pedranzani et al., 2003). Abiotic stress results in both altered levels of phytohormones and decreased plant growth (Moergan, 1990). An alternative strategy to ameliorate salt stress could be, therefore, to use exogenous application of plant growth regulators.

Brassinosteroids (BRs) are steroidal plant hormones implicated in the promotion of
Plant growth and development. Their occurrence has been demonstrated in almost every part of higher plants and green algae *Hydrodictyon reticulatum* and *Chlorella vulgaris* (Bajguz, 2009). Benzyladenine (BA) is one of the naturally occurring cytokinins (implicated in the promotion of plant growth and development) (Nair et al., 2002) and its increasing concentration in crops could be a possible means of reversing the effects of salt stress.

The present study deals with the use of the heterocyclic cytokinin benzyladenine in alleviation of the effects of NaCl-induced stress on growth and metabolic activities of the chlorophycean freshwater alga *Chlorella vulgaris*.

**MATERIAL AND METHODS:**

**Organism and growth conditions:**

*Chlorella vulgaris* Beijerinck MUAC 103 (from the algal collection of Botany Department, Faculty of Science, Mansoura University) was grown in axenic cultures at 22-24°C under continuous illumination (72 μmol photon m⁻² s⁻¹) in 500-ml Erlenmeyer flasks, containing 150 ml MBL media (Nichols, 1973). Test flasks were inoculated with 5-day-old culture with initial cell density 9 x 10⁶ cells ml⁻¹. Growth and metabolism of *Chlorella* cells were studied in response to presence of 10 ppm benzyladenine (BA) and salinization treatments (2, 5 and 15 ppt NaCl).

Growth was followed by determination of specific growth rate (SGR) based on cell count every two days over an experimental period of 12 days (OECD, 2002), measurement of cultures optical density at 670 nm (Alyabyev et al., 2006) and dry biomass of cultures.

**Analytical methods:**

**Photosynthetic pigments:**

Intracellular content of chlorophyll (Chl) a, Chl b and carotenoids (Car.) were extracted using 90% acetone and determined spectrophotometrically according to Wellburn (1994).

**Carbohydrate determination:**

Total soluble sugars and polysaccharides were determined by the anthrone method (Yemm and Willis, 1954).

**Endogenous hormones level:**

Determination of indol acetic acid (IAA), gibberellic acid (GA3), abscisic acid (ABA) were carried out by gas liquid chromatography (GLC) according to Shindy and Smith (1975) and Vogel (1975). Cytokinins were quantified by HPLC (Beckman, System Bold, Programmable Solvent Module 126) according to Muller and Hilgenberg (1986).

**Protein determination:**

Protein concentration was quantified by the method of Bradford (1976).

**Identification and quantitative determination of amino acids composition:**

Amino acid analysis were carried out according to the method of Anderson et al. (1977) using the HPLC.

**Statistical analysis:**

Computations and data analysis were done according to Gomez and Gomez (1984).

**RESULTS AND DISCUSSION:**

The long-term effects of salinity alone and in combination with BA on growth and metabolism of *Chlorella vulgaris* were assessed. Optical density measurements (Fig. 1) as function of time, cell count based specific growth rate “SGR” (Fig. 2), biomass yield (Fig. 3 a&b) and photosynthetic pigments content (Fig. 4 a-d) were performed to determine the effect of different salinities and BA supplementation on growth of *Chlorella* cells. Figures 1-7 indicated a progressive increase in all measured growth criteria with the experimental period. Microalgal growth is characterized by a sigmoid or logistic function (Schanz and Zahler, 1981). In the present study, the growth of *Chlorella* could be fitted to the parameters of a logistic function, except for 15 ppt and 15 ppt + BA stressed cultures (Fig. 1 a & b) which showed a prolonged lag phase reached the stationary phase later than control, low-salinity cultures and BA supplemented cultures.
Specific growth rate (SGR) of the stressed *Chlorella* cultures either alone or with BA addition significantly decreased with increasing salinization except for the 2 ppt-NaCl culture & BA + 2 ppt-NaCl culture which showed promotion of growth (Fig. 2) and consequently the dry biomass yield of *Chlorella* cultures have the same pattern of response (Fig. 3 a & b).

Fig. 2. Effect of BA supplementation (10 ppm) on the specific growth rate (count, day⁻¹) of *Chlorella vulgaris* cultures grown under different concentrations of NaCl (mean ±SE).

Fig. 3. Effect of BA supplementation (10 ppm) on Biomass yield (µg/100 ml culture) of *Chlorella vulgaris* cultures grown under different concentrations of NaCl (mean ±SE).

Masojidek et al. (2000) found that the exposure of the green alga *Chlorococcum* sp. to 0.2M NaCl, results in completely stop in cell division although the biomass of dry weight was increased due to an increase in cell size. The treatment of *Chlorella fusca* cultures with KCl, CaCl₂ & MgCl₂ in concentration relative to NaCl as 1:1, 2:1 & 3:1 promoted cell number and dry mass (Shafea, 2003). In the same context, increasing salinity concentrations induced significant reduction in growth of *Chlorella* cells (Alyabyev et al., 2006). Growth inhibition of *Chlorella* cells may be due to vegetative cells failure to differentiate into autospore mother cells (leading to decreased/zero autospore mother cells percentage) and/or rapid death of all cells (Agrawal and Manisha, 2007). The inhibitory effect of salinity on dry weight of *Chlorella* can be attributed to the reduction in photosynthetic pigments and net assimilation rate (Macler, 1988; Masojidek et al., 2000). Also, this may be due to the decrease in water uptake or ion imbalance and the excessive accumulation of toxic ions such as Na⁺ inside the cells (Ayala and O’leary, 1995; Munns, 2002). They added that Na⁺ may have direct toxic effects through interference with the function of K⁺ as a cofactor of various reaction and its deleterious effects on the structural and functional integrity of membrane as well. The obvious increase in the relative growth rate and consequently the dry biomass yield and cell density of cultures may be due to the role of BA on the regulation of both cell division and enlargement, delaying senescence and gaining growth (Wilkins, 1984). Thus, it can be suggested that *Chlorella* was unable to adapt to...
concentration of 5 and above – a degree of salinity that indicates a critical concentration for the adaptive processes of this cell (Alyabyev et al., 2006). Excess NaCl affected the coupling of light harvesting complex II and photosystem II (LHClII-PSII), but changes in thylakoid architecture and in the PSII assembly state allowed sufficient integrity of the photosynthetic membrane (Ferroni et al., 2007).

The promotive effect of BA noticed on Chlorella growth parameters was coincided with the results of Kapoor and Sharma (1981) who found that kinetin promoted growth of blue-green algae. The increase in dry weight of NaCl-stressed cultures after BA treatments may be attributed to the stimulatory effect of BA on growth through induction of high levels of endogenous phytohormones (Wilkins, 1984).

Long-term salinity treatment induces significant reduction in photosynthetic pigments content either with or without BA supplementation except for low salinized cultures (2 ppt NaCl and BA + 2 ppt NaCl) which exhibit significant increase in pigment content (Figures 4, 5, 6, and 7). Hypo-saline treatments induce significant increases in chlorophyll levels and accumulation of polyamines (Lee and Chen, 1998). Successive increase of total carotenoids levels in stressed cultures and consequently Car./Chl a + Chl b ratio had been significantly increased progressively during the experimental period. The decreased chlorophylls content under salinity is in agreement with the finding of Macler (1988) on the red alga Gelidium coulteri who attributed this effect to the reduction in photosynthetic rate due to salt osmotic and toxic ionic effects. Alyabyev et al. (2006) suggested that an important feature of salt sensitive and salt tolerant microalgae is the increased energetic rate which ensures a quick and effective adaptation of these algae to stress. The obtained significant stimulation in carotenoids content with increasing salinization is in agreement with the findings of Masojdek et al. (2000) on Chlorococcum sp.; and that of Ghezelbash et al. (2008) on the microchlorophyte Tetraselmis chuii.

Fig. 5 (a & b). The content of chlorophyll b (µg / 100 ml culture) of Chlorella vulgaris grown under BA (10 ppm) and different NaCl concentrations (mean ± SE).

Fig. 6 (a & b). The content of carotenoids (µg / 100ml culture) of Chlorella vulgaris grown under BA (10 ppm) and different NaCl concentrations (mean ± SE).

Fig. 7. Carotenoids/chlorophyll a +chlorophyll b ratio of Chlorella vulgaris grown under BA (10 ppm) and different NaCl concentrations (mean ± SE).
The resulted increase in photosynthetic pigments in response to BA supplementation may be due to the role of BA in increasing the endogenous cytokinins and this in accordance with earlier reports demonstrating that cytokinins inhibit the degradation of chlorophylls and photosynthetic proteins as well as chlorophyllase activity, is the major mechanism of delaying plant senescence and increase the chlorophylls content (Czerpak and Bajguz, 1997).

**Carbohydrates fractions:**

The results indicated that low salinization (2 ppt – NaCl) and/or BA addition induced increases in total carbohydrate (Fig. 8) as well as increasing salinization induced significant increases in total soluble sugars (15 ppt NaCl and BA + 15 ppt NaCl). The present results orchestrated with these of Vazquez and Arredodondo (1991) on the chlorophyte Botryococcus braunii. Stress – induced increase in carbohydrate levels in plants is one of the main adaptation mechanisms and osmotic adjustment to salt luminance stress (Ashraf and Harris, 2004). Plants under stress condition accumulate soluble sugars in other words induce starch degradation and polysaccharides synthesis (Kerapesi and Graliba, 2000).

### Endogenous phytohormones:

Increasing salinity of Chlorella vulgaris culture induced remarkable decreases in auxins, gibberellins, cytokinins contents and increase in abscisic acid level. BA addition induced high increases in growth promoters either alone or with salinization (Table 1). Stress may induce common responses such as enhancement of growth inhibitors. Abscisic acid (ABA) accumulation is a key factor in controlling downstream responses essential for adaptation to various stresses. NaCl stress induced sharp changes in the endogenous hormonal balance of Chlorella cell, presented by accumulation of ABA associated with decreased levels of growth promoters, IAA, GA₃ and cytokinins (Wang et al., 2001).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Growth promotors</th>
<th>Growth inhibitor</th>
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<tbody>
<tr>
<td>Control</td>
<td>IAA</td>
<td>GA₃</td>
</tr>
<tr>
<td></td>
<td>17.07</td>
<td>793.93</td>
</tr>
<tr>
<td>NaCl (2 ppt)</td>
<td>18.77</td>
<td>898.57</td>
</tr>
<tr>
<td>(5 ppt)</td>
<td>12.81</td>
<td>755.2</td>
</tr>
<tr>
<td>(15 ppt)</td>
<td>10.56</td>
<td>538.33</td>
</tr>
<tr>
<td>BA (10 ppm)</td>
<td>24.28</td>
<td>814.56</td>
</tr>
<tr>
<td>BA + NaCl (2 ppt)</td>
<td>32.07</td>
<td>807.33</td>
</tr>
<tr>
<td>(5 ppt)</td>
<td>20.73</td>
<td>529.48</td>
</tr>
<tr>
<td>(15 ppt)</td>
<td>13.86</td>
<td>506.14</td>
</tr>
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</table>

Marsalek et al. (1992) indicated that Chlorella vulgaris and Stichococcus bacillaris increase extracellular ABA production under salt, acid and drought stresses. The reduction in auxins content in the current study in response to salinity could be attributed to decrease in IAA biosynthesis and/or to the increase in IAA-oxidation by IAA oxidase enzyme (Bekheta and El-Basiouny, 2005). BA treatments alleviate the adverse effects of salinity. In this context, BA treatment not only increases the extractable active gibberellins, but also increases the synthesis of GA₃O from GA₃ (Goodwine, 1987). Moreover, BA exogenously applied stimulated dihydrozeatin–7 glucoside & zeatin in radish seedlings (Blagove et al., 2004). The decrease in ABA content in the current study may be due to the shift of the common precursor iso-pentyl-pyrophosphate into the biosynthesis of cytokinins and/or gibberellins instead of ABA (Hopkins and Hüner, 2004).

### Protein and amino acids composition:

The increase in the NaCl concentration results in a significant decrease in total soluble protein (TSP) content, except for low salinity culture (2 ppt NaCl either with or without BA) in which TSP increased and culture seemed tolerant to this concentration (Fig. 6). These results are in agreement with those of Macler (1988) on the red alga Gelidium coulteri, Ghezelbash et al. (2008) on the green microalga Tetraselmis chuii, who attributed the increasing in protein in
response to low salinity to the probability of synthesis of new stress proteins. In salt-stressed plants, the decrease in protein level is attributed to: 1- a decrease in protein synthesis, 2- decrease availability of amino acids, and/or 3- denaturation of the enzymes involved in the synthesis of amino acids and proteins (Dubey and Rani, 1990). Increased proteins content by BA treatment may also increase the formation of rough endoplasmic reticulum that provides the appropriate medium for increasing polyribosomes and mRNA (Kaber, 1987).

Acidic amino acids (glutamic and aspartic) and phenylalanine and proline constitute more than 40% of the total amino acid pool of unsalvanized Chlorella vulgaris cells, whereas the lowest amino acid contents were recorded to iso-leucine and histidine (Table 2). Salinization induce high increases, up to 100%, in total amino acids pool in Chlorella cells and/or BA addition which include the increase in total amino acids in values were always higher than their corresponding values in culture with NaCl treatments. Salinity treatments induced high content of protein than control level, but BA addition induce increase in proline content to a level lower than NaCl alone.

It is worthy to mention that cysteine not detected in control and salinized Chlorella cultures; meanwhile it appeared in low salinity cultures (2 ppt NaCl) and/or BA addition. These results are in agreement with that of Abdel Latef et al. (2009). Proline plays an important role in osmoregulation of plant cells (Ahmad and Hellbust, 1988). Production of enzyme stabilization of the protein synthesizing machinery (Kadpal and Rao, 1985), regulation of cytosolic acidity (Venekemp, 1989), acting as an effective singlet oxygen quencher (Alia and Matysik, 1989), acting as an effective enzyme stabilization of the protein synthesizing machinery (Kadpal and Rao, 1985), regulation of cytosolic acidity (Venekemp, 1989), acting as an effective singlet oxygen quencher (Alia and Matysik, 2001). Proline is reported as osmoprotectant in plants subjected to hyposmotic stresses such as drought and soil salinity. The capacity to accumulate proline is correlated with salt tolerance and the increased resistance to oxidation stress is due to some indirect metabolic physiological consequences of the accumulation of proline and other metabolites.

Therefore, in microalgae and other plants proline acts as a free radical scavenging and increases salt tolerance of these organisms (Hong et al., 2000) who suggested a role for proline in reducing oxidative stress induced by osmotic stress, in addition to its accepted role as an osmolyte. It can be concluded from the results that BA addition may play a role in the inhibition effects of salinity and osmoregulate cell metabolism either by producing high contents of the low molecular weight molecules (*LMWM* amino acids, protein and soluble sugars) or enhancing growth promoting substances.

# REFERENCES:


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التأثير المحسن للنزيل إاديبين على طحلب المياه العدبة كلونيللا فولجاريز النامي تحت
تأثير جهاد كورين الصوديوم

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المركز القومي للبحوث - الجيزة - مصر

تعتبر هذه الدراسة أحد دراسات التأثيرات الفسيولوجية والبيوكيميائية الناجحة على تأثير استخدام النزيل (1G) في مطيع (Chlorella fusca) و إضافة مزيل النزيل (LMWM) في مطالعات النباتات فولجاريز النامي تحت تأثير نزيل

الم журнал عن:

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