



Metabolic changes and activity pattern of antioxidant enzymes induced by salicylic acid treatment in green microalga *Dunaliella salina* under nitrogen deficiency

Marzieh Mirshekari¹ · Alireza Einali¹ · Jafar Valizadeh¹

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Abstract

The effect of salicylic acid (SA), an endogenous plant growth regulator, on adaptation of the green microalga, *Dunaliella salina* to N starvation was investigated through the study of enzymatic antioxidant system and biochemical changes. Algal cells in the exponential growth phase were exposed to N deficiency with 100 μ M of SA. N starvation significantly decreased cell number, chlorophyll *a*, and hydrogen peroxide and while highly increased levels of fresh weight, soluble sugars, starch, proteins, free amino acids, and the activity of antioxidant enzymes. N-starved cells treated with SA enhanced cell number and hydrogen peroxide content, but accumulated lower amounts of metabolites and enzymatic activities compared to untreated cultures. However, the levels of fresh weight, chlorophyll, β -carotene, and soluble proteins remained roughly unchanged relative to N starvation alone. Proteolytic activity was well correlated with accumulation of amino acids in control and other treatments. The results suggest that exogenous SA treatment can enhance adaptation to N starvation by establishing the enzymatic balance to adjust levels of metabolites and directing them to the growth processes.

Keywords Antioxidant enzymes · *Dunaliella salina* · Chlorophyceae · N starvation · Protease · Salicylic acid

Introduction

Nitrogen (N) is one of the most important macronutrients required for the proper function of plants and algae. It is a fundamental constituent in the biosynthesis of amino acids, proteins, nucleic acids, enzymes, and photosynthetic pigments (Grossman and Takahashi 2001; Raven and Giordano 2016). Nitrogen deficiency in many ecosystems, especially aquatic ecosystems brings about to growth limitation, which can affect the morphology and physiology of organisms, particularly algae (Syrett 1981; Grossman and Takahashi 2001). One of the important effects of N starvation is decrease in carbon fixation (Einali et al. 2013), which result in damage photosynthetic structures because of reactive oxygen species (ROS) generation even under low light intensity (Asada 1999; Logan et al. 1999; Grossman and Takahashi 2001). Plants have

developed a ROS-scavenging system consists of antioxidant molecules, such as salicylate, ascorbate, tocopherols, carotenoids, and antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), and pyrogallol peroxidase (PPX) (Foyer and Noctor 2003; Sairam and Tyagi 2004), to maintain cellular structure. Increase in the activities of ROS-scavenging systems in response to various biotic and abiotic stresses has been previously reported (Logan et al. 1999; Grant and Loake 2000; Haghjou et al. 2009). In addition, stress adaptation can be achieved by metabolic adjustments, which is associated with accumulation of osmolytes and several organic solutes (Mishra and Jha 2011).

Salicylic acid (SA) is an endogenous phytohormone with a simple phenolic structure, which plays a key role in the regulation of many physiological processes (Karlidag et al. 2009; Hayat et al. 2010). The role of SA in plant resistance to biotic and abiotic stress including pathogens attacks (Enyedi et al. 1992; Alverez 2000), salinity, drought, ultraviolet radiation (Hua et al. 2008) and heavy metal stress (Mishra and Choudhuri 1999) has been demonstrated. It has been recently demonstrated that SA is able to protect peanut against oxidative stress induced

✉ Alireza Einali
aeinali@science.usb.ac.ir

¹ Department of Biology, Faculty of Science, University of Sistan and Baluchestan, Zahedan, Iran

by iron deficiency (Kong et al. 2014). Various studies have demonstrated that exogenous application of some signal molecules and antioxidants including salicylic acid, jasmonic acid, and ascorbate induce the activity of antioxidant systems (Li et al. 1998; He et al. 2002; Athar et al. 2008; Saruhan et al. 2012) and enhance synthesis or accumulation of soluble proteins and free proline (Ashraf and Foolad 2007; Nikolaeva et al. 2010), thereby improving abiotic stress tolerance in plants. However, the effects of antioxidant molecules on algal tolerance to abiotic stress have been rarely considered.

Several members of the green algal genus *Dunaliella* are extraordinary organisms because of their viability in various extreme environmental conditions (Rothschild and Mancinelli 2001). *Dunaliella salina* is considered as an interesting model system for stress physiology studies because of high physiological flexibility and tolerance under unfavorable conditions, especially high salt concentrations (Avron and Ben-Amotz 1992; Cowan et al. 1992; Borowitzka 2013). Under different stress conditions, or even naturally, some species of *Dunaliella* accumulate high levels of β -carotene (Ben-Amotz and Avron 1983; Cowan et al. 1992; Salguero et al. 2003). *Dunaliella salina* is a commercial source of natural β -carotene (Borowitzka 2013). The amount of β -carotene production in this alga under growth-limiting conditions such as high salinity, high irradiance, high temperature, and limiting nutrients is estimated to more than 10% of its dry weight (Ben-Amotz et al. 1982; Ben-Amotz and Avron 1983; Cowan et al. 1992; Salguero et al. 2003; Borowitzka 2013). β -carotene acts as a ROS-scavenger molecule by quickly quenching singlet oxygen and free radicals such as superoxide radicals and lipid peroxy (Krinsky and Yeum 2003) and thus protect chlorophyll against photo-damage (Ben-Amotz and Avron 1983; Ben-Amotz et al. 1989). Because this alga naturally produces high levels of β -carotene under stress conditions (Ben-Amotz and Avron 1983; Cowan et al. 1992; Salguero et al. 2003), it can be assumed to be a response of this alga to stress. However, there is no difference between high and low β -carotene *Dunaliella* cells in defense susceptibility against various oxidants, indicating that other options such as different antioxidants and or metabolic modulations may also be involved in stress adaptation in *Dunaliella* (Jimenez and Pick 1993; Haghjou et al. 2009; Mishra and Jha 2011). Despite extensive studies on the role of SA in plant tolerance to abiotic stress, its possible interactive effects and stress on antioxidant system activity and biochemical changes in *Dunaliella* cells have not been investigated yet. The aim of the present work was to assess the effects of SA on growth, biochemical changes and the antioxidant enzymes activity in *D. salina* under N deficiency.

Materials and methods

Algal cultures and experimental treatments

Dunaliella salina Teod. (UTEX 200) was obtained from The Culture Collection of Algae at the University of Texas at Austin, USA. The cells were grown in a modified Johnson medium (pH = 7.5) containing 5 mM KNO₃, 5 mM MgSO₄, 7 μ M MnCl₂, 1 μ M CuCl₂, 1 μ M CoCl₂, 1 μ M ZnCl₂, 1 μ M Na₂MoO₄·2H₂O, 0.2 mM CaCl₂, 0.2 mM KH₂PO₄, 4 μ M FeCl₃, 10 μ M Na₂-EDTA, and 24 mM NaHCO₃ with 1.5 M NaCl (Einali and Valizadeh 2015). Suspensions were incubated in a culture room at 25 °C under continuous shaking at 100 rpm with a photoregime of 16 h light (70 μ mol photons m⁻² s⁻¹)/8 h dark. When the suspensions reached the phase of logarithmic growth, they were collected by centrifugation at 3000×g for 5 min and resuspended in fresh N-containing (NC) or N-deficient (ND) medium. Each suspension (NC or ND) was separated into two flasks. One of each divided suspensions was treated with 0.1 mM SA (SA or ND + SA) and another remained untreated. All cultures were incubated under the growth conditions described above. Three separate flasks were provided for each experiment, and sampled 48 h after SA or N deficiency treatment.

Determination of cell number, optical density and fresh weight

Algal cells were enumerated using a hemocytometer (Schoen 1988). Cell optical density was determined spectrophotometrically as absorption at 680 nm against free cell culture medium. Thirty milliliters of algal suspensions were harvested by centrifugation at 2000×g for 10 min to determine fresh weight. Algal pellet was resuspended in a small volume of its supernatant and poured into a pre-weighed micro-tube and recollected by centrifugation at 10,000×g for 5 min. Prior to weighing the resultant pellet, it was washed with fresh culture medium containing 0.2 M NaCl to remove residual salt (Haghjou et al. 2014).

Pigment determination

Pigments determination including chlorophyll (Chl) and β -carotene was carried out by precipitating 1 mL of cell suspension at 10,000×g for 5 min and extracting with 1 mL of 80% (v/v) acetone. The mixture was centrifuged at 10,000×g for 2 min. Chl content was determined spectrophotometrically according to the method of Arnon (1949). β -carotene concentration was calculated using $E_{1\text{cm}}^{1\%}$ of 2273 at 480 nm (Ben-Amotz and Avron 1983).

Extraction and measurement of total soluble sugar and starch

For extraction of soluble sugars and starch, weighed pellets (obtained from 30 mL of cell suspension as described above) were mixed with 1 mL of pure acetone and centrifuged at $10,000\times g$ for 2 min. The supernatant was removed and this action was repeated two more times to completely remove the pigments. The resultant decolorized pellet was extracted with 5 mL of 80% (v/v) ethanol at 70 °C for 10 min. After centrifugation at $2000\times g$ for 10 min, the supernatant was saved and the pellet re-extracted four times with the same volume of 80% ethanol. These ethanolic extracts were pooled and concentrated to one sixth of their original volume by evaporation, and used for determination of total soluble sugars levels according to the anthrone method of McCready et al. (1950) using glucose as the standard.

Starch was extracted and determined according to the method of McCready et al. (1950). The pellet after final soluble sugar extraction was mixed with 0.2 mL of distilled water and 0.26 mL of 52% perchloric acid and incubated in an ice bath for 15 min. Distilled water to the volume of 0.4 mL was added to the mixture and centrifuged at $2000\times g$ for 10 min. The supernatant was saved in ice, and the pellet was re-extracted with 0.1 mL of distilled water and 0.13 mL of 52% perchloric acid. The extracts were combined and reached final volume of 2 mL with distilled water. Total soluble sugar concentration results from starch breakdown were quantified, and starch content was determined by multiplying the glucose content by the glucose equivalent of 0.9.

Total protein and free amino acid determination

Total protein was extracted from the decolorized pellet according to the method of Stone and Gifford (1997) with some modifications. Briefly, the algal decolorized pellet was extracted with 0.5 mL of sample buffer (60 mM Tris-HCl buffer (pH 6.8), 10% (v/v) glycerol, and 2% (w/v) SDS) at 90 °C for 1 h. The mixture was centrifuged at $10,000\times g$ for 15 min and the supernatant was used for determination of total protein content. The Markwell et al. (1981) method was used to determine total protein amount due to SDS interference in the Bradford (1976) protein assay.

Free amino acids extraction was carried out as described for soluble sugar except that the fresh weighed pellet (obtained from 30 mL of cell suspension) used instead of decolorized pellet. Depigmentation of the concentrated ethanolic extract was carried out using chloroform (1:5; v/v). Total free amino acid content in the aqueous phase was determined according to the ninhydrin method of Yemm and Cocking (1955) using glycine as the standard.

Enzyme extraction and assay

Enzymes were extracted from the weighed pellet (obtained from 30 mL of cell suspension as described above) with 1 mL of enzyme extraction buffer containing 100 mM cold potassium phosphate buffer (pH 7.0), 10% glycerol, 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 70 mM 2-mercaptoethanol, 0.1% (v/v) Triton X-100, and 1% (w/v) polyvinylpyrrolidone (PVPP). The extraction buffer also contained 5 mM ascorbic acid when extracting ascorbate peroxidase (APX). For extraction of algal protease(s), EDTA was excluded from the extraction buffer (Einali and Valizadeh 2017). The cells were broken by ultrasonic for two cycles of 30 s with 2-min interval and afterwards incubated for 1 h at 4 °C. Decolorization of the suspension was carried out by adding 10 mg of charcoal followed by centrifugation at $12,000\times g$ for 10 min at 4 °C. The resultant clear supernatant (soluble protein fraction) was used to determine enzymes activity. Total soluble protein (TSP) content was quantified according to the method of Bradford using bovine serum albumin (BSA) as the standard (Bradford 1976).

CAT activity was assayed by the method of Luck (1965). One milliliter of reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 12.5 mM H_2O_2 , and enzyme extract. The rate of H_2O_2 breakdown was calculated at 240 nm using the extinction coefficient of $0.0394\text{ cm}^2\ \mu\text{mol}^{-1}$.

APX activity was determined according to the method of Chen and Asada (1992). One milliliter of reaction mixture composed of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 1 mM H_2O_2 , and enzyme extract. The rate of dehydroascorbate production was measured at 290 nm using the extinction coefficient of $2.8\text{ cm}^2\ \mu\text{mol}^{-1}$.

Pyrogallol peroxidase (PPX) and polyphenol oxidase (PPO) activity was assayed according to the method of Nakano and Asada (1981). For PPX, 1 mL of reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM H_2O_2 , 40 mM pyrogallol, and enzyme extract. The reaction mixture is the same for PPO, with the difference that H_2O_2 was excluded. The increase in the absorbance due to purpurogallin production was measured at 430 nm using the extinction coefficient of $2.47\text{ cm}^2\ \mu\text{mol}^{-1}$.

The proteolytic activity in algal suspensions was determined by measuring the rate of release of soluble amino nitrogen as described by Peoples and Dalling (1978) with some modifications. The reaction mixture containing 0.5 mL of substrate (1% bovine serum albumin (BSA) in 50 mM phosphate buffer (pH 4.5, 7.5, 9)), 0.1% (v/v) 2-mercaptoethanol, and 0.1 mL of enzyme extract was incubated for 2 h at 37 °C. The reaction was terminated by adding 0.7 mL of 15% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged at $5000g$ for 10 min, and the TCA-soluble nitrogen was determined according to the ninhydrin method of Yemm and Cocking (1955) using glycine as the standard.

Determination of hydrogen peroxide

Hydrogen peroxide (H_2O_2) was extracted from the fresh weighed pellet (obtained from 30 mL of cell suspension) with 5 mL of 0.1% (w/v) cold TCA in an ice bath. The cells were broken by ultrasonic twice for 30 s with 2-min interval. The homogenate was then centrifuged at $12,000\times g$ for 15 min and the supernatant was used for determination of H_2O_2 content by the method of Alexieva et al. (2001). The reaction mixture contained 0.5 mL of 100 mM potassium phosphate buffer (pH 7.4), 0.5 mL of the supernatant, and 2 mL of 1 M potassium iodide. The mixtures were held for 1 h at room temperature in the dark and their absorbance recorded at 390 nm. Hydrogen peroxide content was estimated using an H_2O_2 calibration curve.

Statistical analysis

All data are reported as the mean and standard deviation (SD) of three independent measurements from separate flasks. Statistically significant differences between treatments were determined at $P < 0.05$ using two-way factorial analysis of variance (ANOVA) with a Tukey multiple comparisons post hoc test. Equal variance and normality tests were carried out before ANOVA.

Results

The effect of treatments on cell number and fresh weight

The number of cells significantly decreased after 48 h in N-deficient suspensions (ND) in relation to control (NC) (Fig. 1a). SA treatment alone showed no significant change in cell number after 48 h, while the number of cells had a significant increase in SA-treated cells under N deficiency (ND + SA) (Fig. 1a).

The fresh weight of cells was significantly increased after 48 h in response to N deficiency (Fig. 1b). The fresh weight of cells treated with SA did not significantly change relative to untreated cultures. However, in SA treatment alone, the fresh weight of cells was significantly lower in comparison with combined SA treatment and N deficiency (Fig. 1b).

The effect of treatments on pigments content

Chl *a* and total Chl contents were significantly decreased, while Chl *b* content remained unchanged in untreated algal cells grown under ND after 48 h with reference to NC (Fig. 2a–c). The Chl *a*, Chl *b*, and total Chl contents of suspension treated with SA alone was significantly higher

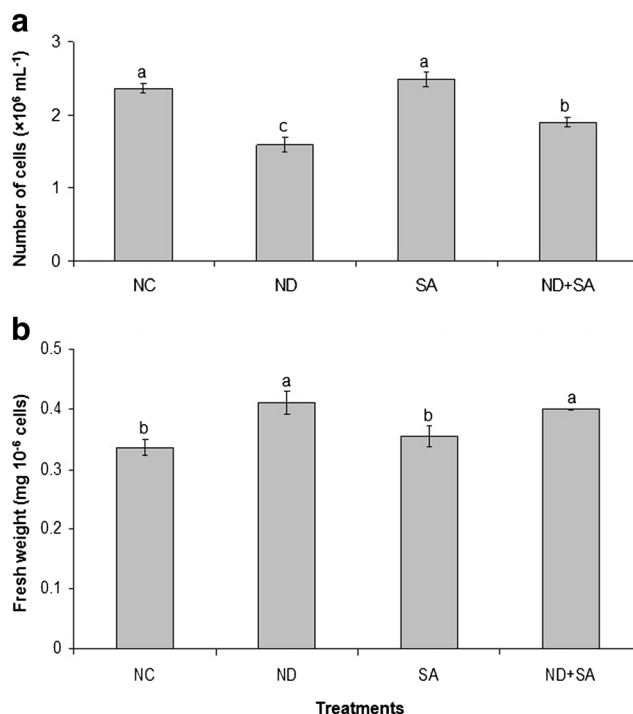


Fig. 1 Number of cells (a) and fresh weight (b) of *Dunaliella salina* cells under N deficiency with or without 100 μM of SA after 48 h. Values are the mean \pm SD of three separate experiments and different letters show significant differences between the various treatments at $P < 0.05$ according to the Tukey test

than untreated control, but they were unchanged in SA-treated cells under N-deficient conditions (Fig. 2a–c). The Chl *a/b* ratio significantly decreased both in algal cells grown under ND and in response to SA treatment alone in relation to NC, while it was unaltered in ND + SA with respect to cultures grown under N deficiency alone (Fig. 2d).

In contrast to Chl, β -carotene concentration did not change in untreated algal cells grown under ND after 48 h in relation to NC (Fig. 2e). SA treatment alone in contrast to combination of SA with N-deficient conditions showed a significant increase in β -carotene content (Fig. 2e). N deficiency either alone or in combination with SA treatment displayed a positive change in the β -carotene to total Chl ratio, but the ratio remained unchanged in response to SA treatment alone as compared to control (Fig. 2f).

The effect of treatments on total soluble sugar and starch contents

In response to N deficiency, both total soluble sugar and starch contents were significantly increased (Fig. 3). Soluble sugar content of algal cells was positively changed by SA treatment. However, it had a significant decrease in SA treatment combined with N deficiency

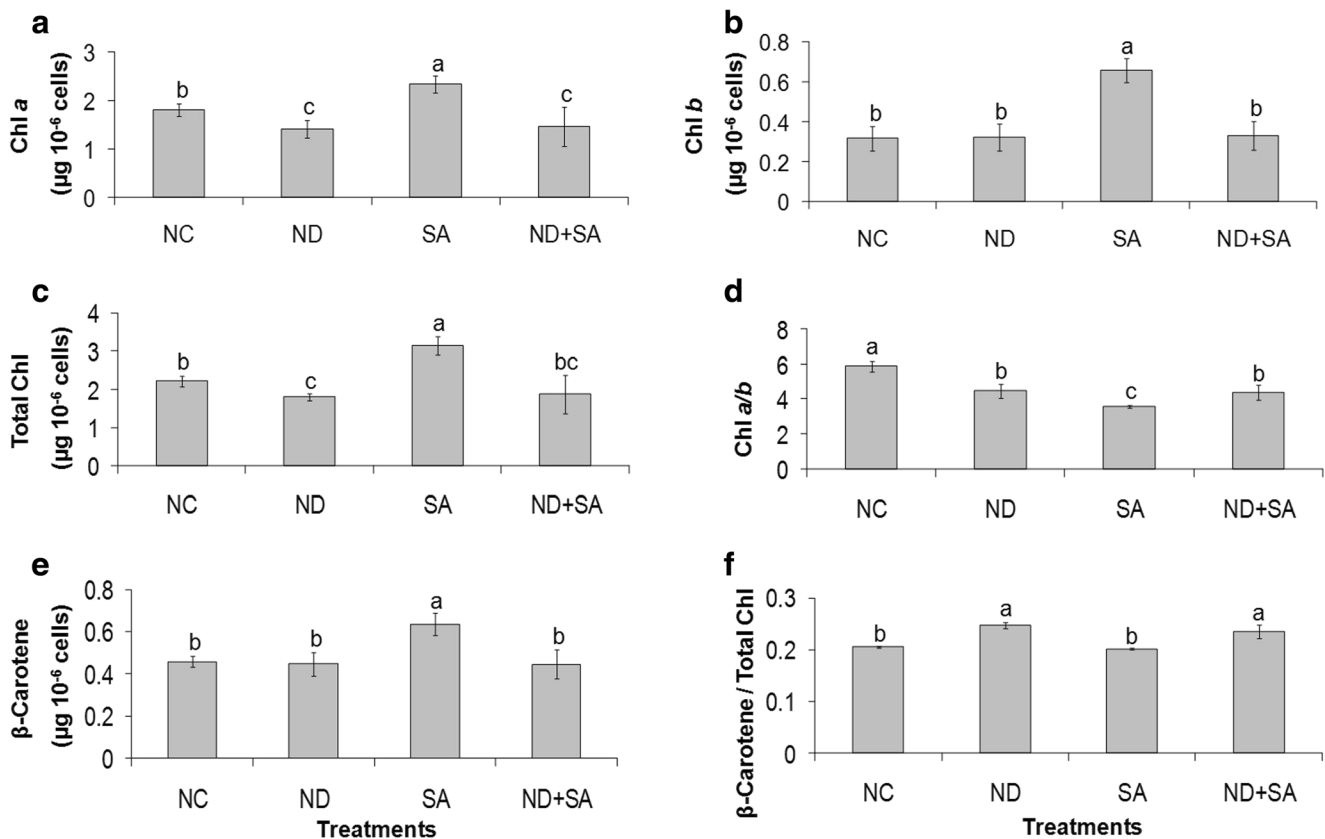


Fig. 2 Photosynthetic pigments content of *Dunaliella salina* under N deficiency with or without 100 μM of SA after 48 h. **a** Chl a, **b** Chl b, **c** total Chl, **d** Chl a/b ratio, **e** β -carotene concentration, and **f** ratio of β -

carotene to total Chl. Each value is mean \pm SD of three independent experiments. Different letters indicate significant differences between the various treatments at $P < 0.05$ according to the Tukey test

relative to the untreated ND (Fig. 3a). SA treatment alone did not change starch content of algal cells, but in combination with N deficiency did result in decreased starch with respect to the cells grown in N deficiency alone (Fig. 3b).

The effect of treatments on antioxidant enzymes activity

The activity of all four antioxidant enzymes (i.e., CAT, APX, PPX, and PPO) of N-depleted cells was highly increased after 48 h relative to control (Fig. 4). CAT activity was positively changed by SA treatment alone, but SA treatment along with N deficiency significantly decreased CAT activity relative to the untreated N-depleted cells (Fig. 4a). APX and PPX activities of both SA-treated cultures were significantly decreased compared to untreated cultures (Fig. 4b, c). PPO activity of algal cells was not affected by SA treatment alone, but the enzyme activity had significant decreased in combination of SA and N deficiency with reference to the untreated N-depleted cells (Fig. 4d).

Changes in hydrogen peroxide content in response to SA

H_2O_2 content of N-deficient suspension was significantly decreased after 48 h in relation to control (Fig. 4e). SA showed a highly positive effect on H_2O_2 content either alone or combined with N deficiency with respect to the untreated cultures.

The effect of treatments on soluble protein, total protein, and amino acid contents

Total soluble protein, total protein and free amino acids levels were significantly increased after 48 h in N-deficient suspensions (ND) in relation to control (NC) (Fig. 5). Total soluble protein refers to proteins that were extracted by extraction buffer in the absence of SDS. Soluble protein level was positively affected by SA treatment alone. However, SA treatment along with N deficiency showed no significant change in soluble protein concentration as compared to the N deficiency alone (Fig. 5a).

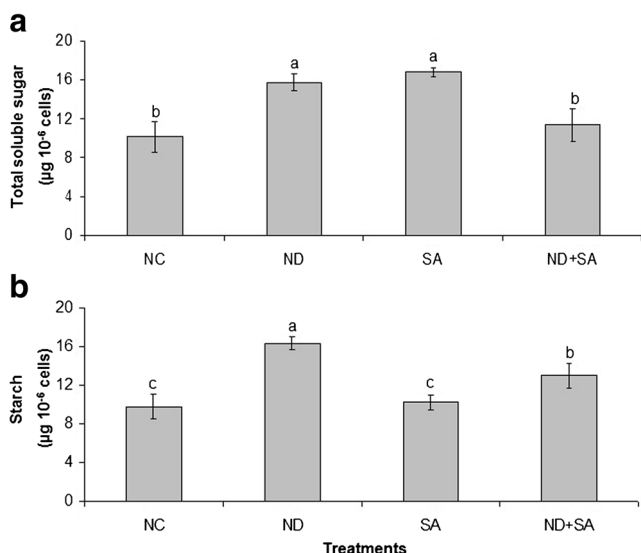


Fig. 3 Changes in total sugars (a) and starch (b) contents of *Dunaliella salina* cells under N deficiency with or without 100 μ M of SA after 48 h. Results are the mean \pm SD of three distinct experiments and different letters exhibit significant differences between the various treatments at $P < 0.05$ according to the Tukey test

The purpose of total protein is the proteins that were extracted by sample buffer containing SDS. Although it is mentioned that SDS does not extract all proteins (Einali and Sadeghipour 2007), the level of algal proteins known as total proteins showed a massive increase compared with total soluble proteins. This increase was about fivefold for SA treatment alone, while it was found to be more than tenfold for control and other treatments (Fig. 5a, b). SA treatment alone had no significant effect on total protein level with reference to the control. However, combined SA and N deficiency showed a significant decrease in total protein contents relative to N deficiency alone (Fig. 5b). Total free amino acid content of both SA-incubated cultures was drastically decreased compared to non-incubated cultures (Fig. 5c).

Proteolytic activity of *D. salina* cells in response to SA and N-deficiency

Protease activity assay at different pHs revealed that there is a similar pattern of proteolytic activity in both N-containing and N-deficient cultures whether they had been treated with SA or not (Fig. 5d). Generally, the protease activity was greater at acid pH than other pHs in both cultures either incubated with or without SA. Proteolytic activity of N-depleted cells was significantly increased at both acid and alkaline pH with reference to the control, while it remained roughly unchanged at neutral pH. SA treatment either alone or with N deficiency significantly decreased the proteolytic activity at all pHs compared with untreated cultures. However, the decreased proteolytic activity in both SA-treated cultures was more at acid compared to neutral and alkaline pHs (Fig. 5d).

Discussion

The cell growth of N-deficient cultures was negatively affected relative to the control, which emphasize the role of N in vigorous cell division of algal cultures. Exogenous SA treatment improved N deficiency-induced growth decrease, which can indicate the positive effect of SA under N deficiency. Stimulation of cell division by low levels of SA was also found in the apical meristem of wheat seedlings after a short-term effect of salt stress (Shakirova et al. 2003) or in *Arabidopsis* seedlings (Zhou et al. 1998; Vanacker et al. 2001) through inducing the expression of CDC2b, a protein that induces dividing cells as well as those competent to divide, and interacting of hormone with multiple receptors or signaling pathways during stress situations. It is a well-known fact that SA possibly generates a wide range of metabolic responses and induces tolerance against various abiotic stresses to the plants (Hayat et al. 2010). However, increased fresh weight caused by N deficiency in spite of a decrease in cell division may indicate that the algal cells respond to N deficiency by accumulating of some metabolites including soluble sugar, starch, and amino acids. Increased fresh weight of cells caused by starch accumulation was reported earlier (Tian and Yu 2009; Garcia-Gomez et al. 2012). Despite the lower cell growth, an increase in fresh weight of *Dunaliella tertiolecta* cells exposed to a mixture of photosynthetically active radiation, UVA, and UVB was found because of starch accumulation (Garcia-Gomez et al. 2012). Increased fresh weight of cells in *Dunaliella* sp. may also be due to lipids accumulation induced by oxidative stress (Yilancioglu et al. 2014). However, fresh weight of *D. salina* cells did not change in N-deficient culture treated with SA against N deficiency treatment alone, showing that SA is ineffective in fresh weight increase because of enhanced cell division. Although previous studies have revealed the involvement of salicylic acid in cell cycle and enlargement (Riou-Khamlichi et al. 1999; Vanacker et al. 2001; Xia et al. 2009), few data are available regarding the role of SA in the regulation of growth under N deficiency. In contrast, an increase in fresh weight along with a decrease in the number of cells has been previously found under stress conditions in *Dunaliella viridis* cells treated with menadione (Haghjou et al. 2014) and *Dunaliella bardawil* (= *D. salina*) cells treated with propyl gallate (Einali 2018). It can be attributed to the role of SA in enhancing cell division under N-deficient conditions.

N deficiency caused a significant decrease in Chl contents. Despite the positive effects of SA treatment alone on the pigments content, SA treatment combined with N deficiency did not change pigment contents relative to N deficiency alone. Increased pigments content in response to SA treatment alone can be on account of the interaction of SA and available N in algal cells, and that N is necessary to SA function in increasing pigments content.

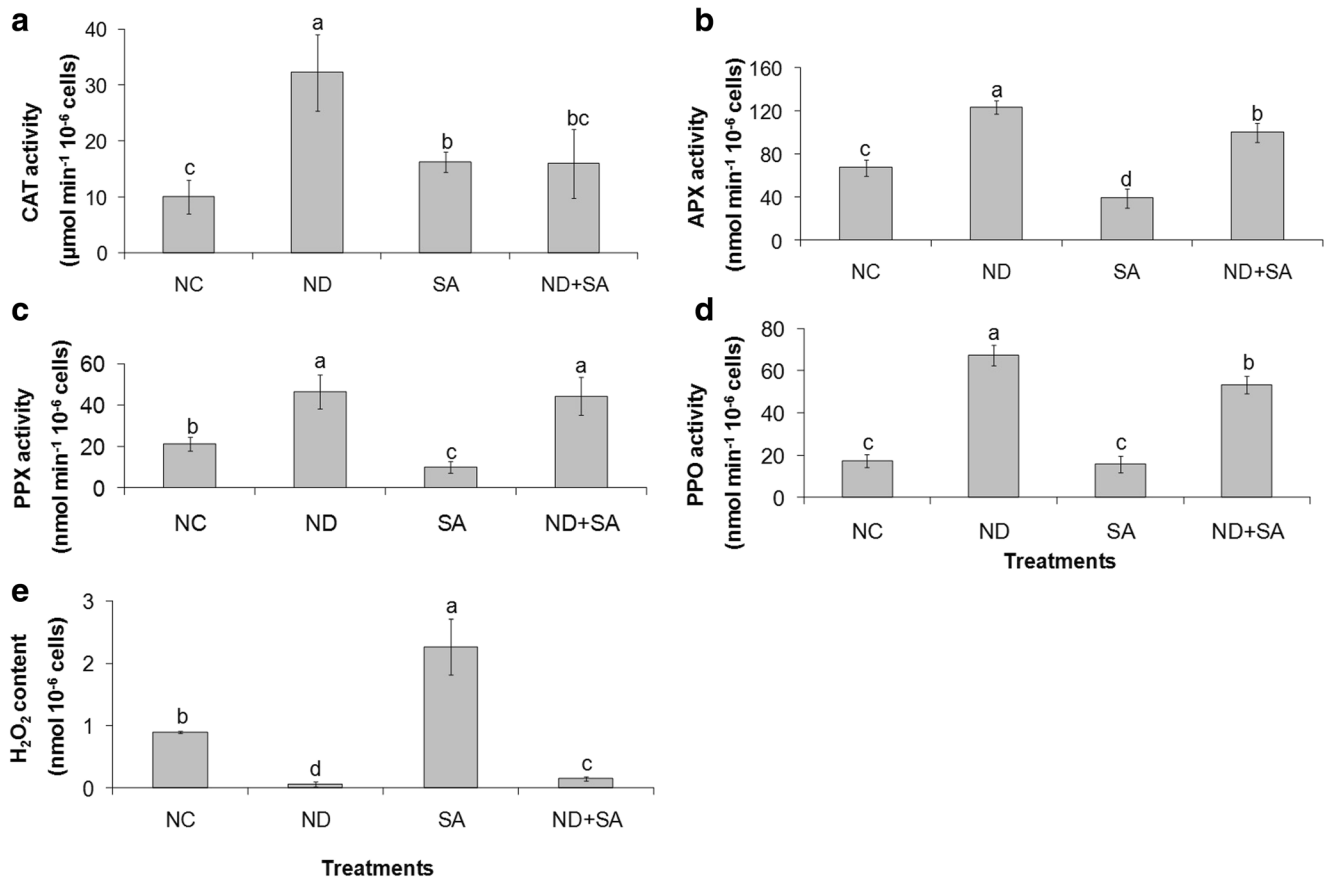


Fig. 4 Changes in activity of CAT (a), APX (b), PPX (c), PPO (d), and H₂O₂ contents (e) of *Dunaliella salina* cells under N deficiency with or without 100 μM of SA after 48 h. Each column is the mean ± SD of three

separate experiments. Different letters reveal significant differences between the various treatments at *P* < 0.05 according to the Tukey test

Evidence to support this suggestion comes from a study showing that exogenous application of SA can increase Chl biosynthesis and N use efficiency through inducing nitrate reductase activity in germinating cucumber seeds (Singh et al. 2010). However, decreased Chl *a/b* ratio in the cells treated with SA alone indicates that Chl *b* is synthesized at a higher rate than Chl *a*. It can be attributed to the central role of Chl *b* in assembling stable light-harvesting complexes through induction of some individual thylakoid membrane proteins, which leads to an increase in the electron flow rates and the antenna complex size (Tanaka et al. 2001; Eggink et al. 2004; Biswal et al. 2012). Synthesis of Chl *b* takes place through oxidation of a methyl group on the B ring of Chl *a* molecule to a formyl group (Porra et al. 1994). Thus, it may be supposed that the enzyme(s) involved in biosynthesis of Chl *b* is upregulated by SA treatment in *D. salina* cells grown in N-containing medium. This is in agreement with our previous study on the role of propyl gallate in *D. salina* cells under salt stress (Einali and valizadeh 2015).

β-carotene concentration did not change under N deficiency alone or in combination with SA treatment, but

increased as a result of treatment with SA alone in relation to the control. It is well-known that *Dunaliella* cells can potentially accumulate large amount of β-carotene under stress conditions even though cell division is decelerate (Ben-Amotz et al. 1982; Jahnke and White 2003; Mishra et al. 2008; Mishra and Jha 2011; Einali 2018). Thus, the lack of change in β-carotene content in N-deficient conditions may be resulting from a slight decrease in β-carotene biosynthesis along with reduced cell division. This is reflected in β-carotene to total Chl ratio, which is considerably more in N-deficient conditions respect to the control, due to unchanging β-carotene against reduced total Chl content. It is in agreement to previous studies indicating that under unstable environmental conditions, increase in the ratio of β-carotene to total Chl is inversely proportional to the growth rate (Ben-Amotz et al. 1982; Ben-Amotz and Avron 1983; Einali 2018).

N deficiency induces high accumulation of metabolites including soluble sugar, starch, free amino acids and protein contents in *D. salina* cells. Sugar accumulation in plant tissues under N deficiency is found in other species (Paul and Foyer

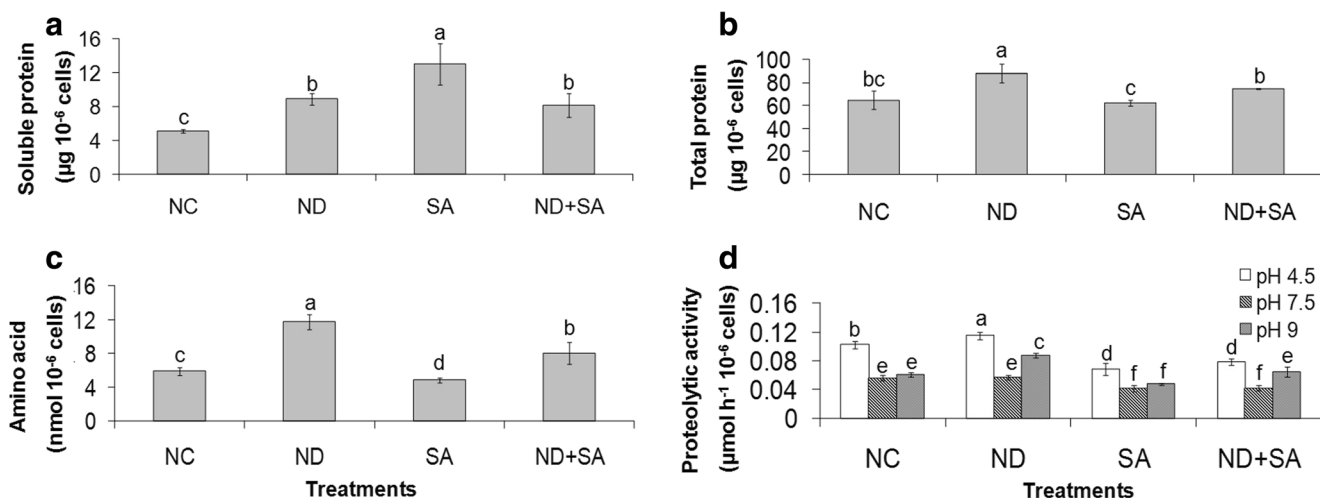


Fig. 5 Changes in total soluble protein (a), total protein (b), amino acid (c) contents and proteolytic activity (d) of *Dunaliella salina* cells under N deficiency with or without 100 μM of SA after 48 h. Results represent

mean ± SD of three independent measurements. Different letters indicate significant differences at $P < 0.05$ between the various treatments according to the Tukey test

2001; Terce-Laforgue et al. 2004). Increased soluble sugar and starch contents in N-depleted cells may be due to declined demand for carbon because of slowing down cell division. Since N is necessary to cell division, lack of coordination between carbon fixation and nitrogen assimilation results in carbohydrate accumulation (Lemaitre et al. 2008). Although feedback down regulation of photosynthesis occurs under N deficiency, the accumulation of high levels of soluble sugars and starch can damage chloroplast structure and leads to lower Chl concentration and CO₂ assimilation (Cave et al. 1981), which is consistent with our results. Thus, the decrease of photosynthesis in N-deficient culture is a determinant factor in the accumulation of soluble sugars and starch, because they both apply the metabolite feedback setting (Koch 2004; Blasing et al. 2005). Reducing the accumulation of soluble sugars and starch in N-deficient culture treated with SA compared to N deficiency alone can be attributed to the elevating demand for carbon in consequence of enhancing cell proliferation in the presence of SA.

Increased proteins and free amino acid contents as a result of N deficiency may show an increase in protein turnover. The importance of the increased protein turnover under N-deficient conditions has been reported earlier (Humphrey et al. 1977). It is because of the imbalance between enzymes under N deficiency. Therefore, the increased protein turnover is the mechanism that thereby a relative balance against the enzyme changing is obtained. SA treatment under N deficiency leads to reduction in proteins and free amino acid contents, perhaps because of the relative balance between the enzymes. This is reflected in the proteolytic activity in response to SA treatment and N starvation. Proteolytic activity assay showed that more active proteases at acid and alkaline instead of neutral pHs may have a role in protein rearrangement and amino acid

accumulation in *D. salina* cells under N deficiency. However, proteolytic activity of SA-treated cultures with or without N revealed a significant decrease at all pHs in relation to untreated cultures, which was well correlated with amino acid accumulation. The association of proteolytic activity with free amino acids accumulation in all algal cultures including control or treatments can further support the suggestion. However, SA treatment alone caused a significant increase in soluble protein associated with a reduction in amino acid content, which can be indicative positive role of SA in protein synthesis. It is consistent with the findings on the role of SA in induction of protein synthesis in plants such as wheat (Kang et al. 2012), barley (Mutlu et al. 2013), and soybean (Razmi et al. 2017).

H₂O₂ and other ROS generation is one of the damaging effects of environmental stresses, which induces oxidative damage to the membrane biomolecules (Munne-Bosch and Penuelas 2003; Gill and Tuteja 2010). In our experiments, H₂O₂ content had significantly decreased in N-depleted cells relative to the control. It can be due to a highly increase in the activity of H₂O₂-decomposing enzymes including APX, CAT, and PPX under N deficiency. Increased the activity of antioxidant enzymes in *Dunaliella* cells exposed to different environmental stresses has also been previously reported (Jahnke and White 2003; Haghjou et al. 2009; Mishra and Jha 2011; Einali 2018). However, SA treatment either alone or combined with N deficiency significantly increased H₂O₂ level compared with untreated cultures, which was well correlated with the reduced activity of the most antioxidant enzymes. There is evidence in the literature that SA induces the generation of H₂O₂ and other ROS (Rao et al. 1997; Shirasu et al. 1997; Minibaeva et al. 2001; Kadioglu et al. 2011). It has been demonstrated that SA-induced H₂O₂ accumulation is

associated with the decrease of APX and CAT activities (Dat et al. 2000; Landberg and Greger 2002). In agreement with the reports, our results showed that accumulation of H₂O₂ induced by SA under N deficiency was associated with a reduction in the activities of CAT, APX, and PPX. SA-induced H₂O₂ accumulation whether in N-containing or in N-deficient cultures may indicate a signaling role for H₂O₂ during adaptation to stress conditions or increased systemic resistance to pathogens, as described before (Dat et al. 2000; Kadioglu et al. 2011). However, PPO activity, as an H₂O₂-independent antioxidant enzyme showed the same activity pattern of H₂O₂-decomposing enzymes in response to SA treatment and N deficiency, indicating that mechanisms other than ROS scavenging may be involved in alleviating the negative effects of result from N starvation by SA treatment in *D. salina* cells. This finding is consistent with our previous study on action mechanism of propyl gallate in tolerance of *D. salina* cells to salt stress (Einali and Valizadeh 2015).

In this study, N starvation significantly decreased growth and Chl synthesis, which was associated with enhanced activity of ROS-scavenging enzymes and accumulation of metabolites such as soluble sugars, proteins, free amino acids, and starch. Our results showed that exogenous SA treatment can increase cell growth and consequently enhance tolerance of *D. salina* cells to N deficiency, but it was accompanied by a significant decline of antioxidant enzymes activity as well as metabolites content. Therefore, the induction of N starvation tolerance by exogenous SA treatment in *D. salina* cells can be achieved through mechanisms other than enhancing the antioxidant system and metabolic adjustment. It seems that SA ameliorates N deficiency tolerance through adjusting activity of enzymes involved in metabolites accumulation and consequently directing the carbon flux to the growth processes.

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