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Research article

Salt induced oxidative stress alters physiological, biochemical and metabolomic responses of green microalga *Chlamydomonas reinhardtii*

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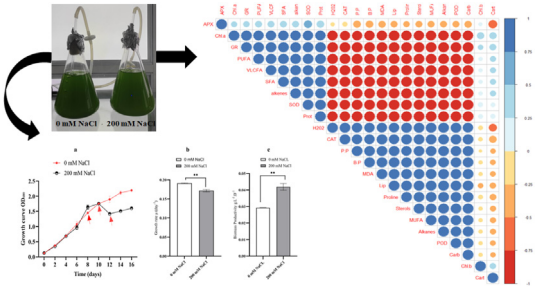
HIGHLIGHTS

- Salinity stress of 200 mM NaCl promoted ROS generation in *C. reinhardtii.*
- ROS induced physiological, biochemical and metabolomic changes in microalga.
- *C. reinhardtii* showed the implication of antioxidative system against salinity stress.
- ROS-Scavenger molecules and enzymes induce resistance to salt stress.
- *C. reinhardtii* revealed the accumulation of metabolites involved in resistance to salt stress.

GRAPHICAL ABSTRACT



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ABSTRACT

Salinity is one of the most significant environmental factors limiting microalgal biomass productivity. In the present study, the model microalga *Chlamydomonas reinhardtii* (*C. reinhardtii*) was exposed to 200 mM NaCl for eight days to explore the physiological, biochemical and metabolomic changes. *C. reinhradtii* exhibited a significant decrease in growth rate, and Chl a and Chl b levels. 200 mM NaCl induced ROS generation in *C. reinhardtii* with increase in H_2O_2 content. This caused lipid peroxidation with increase in MDA levels. *C. reinhardtii* also exhibited an increase in carbohydrate and lipid accumulation under 200 mM NaCl conditions as storage molecules in cells to maintain microalgal survival. In addition, NaCl stress increased the content of carotenoids, polyphenols and osmoprotectant molecules such as proline. SOD and APX activities decreased, while ROS-scavenger enzymes (POD and CAT) decreased. Metabolomic response showed an accumulation of the major molecules implicated in membrane remodelling and stress resistance such oleic acid (40.29%), linolenic acid (19.29%), alkanes, alkenes and phytosterols. The present study indicates the physiological, biochemical and metabolomic responses of *C. reinhardtii* to salt stress.

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1. Introduction

Salt stress is one of the major environmental factors affecting physiological and biochemical pathways related to the growth and development of microalgae (Zhang et al., 2018; Ismaiel et al., 2018). High salinity levels induce ionic, osmotic, and oxidative stress (Qiao et al., 2021). Microalgae, under salt stress, produce various reactive oxygen species (ROS) including hydrogen peroxide, hydroxyl radicals, and singlet oxygen (Yun et al., 2019; Ma et al., 2020).

ROS act as secondary messengers in intracellular signalling cascades that trigger various abiotic and biotic stress adaptative responses. However, high ROS accumulation can be damaging macromolecules (proteins, lipids and nucleic acids), affecting cellular metabolism and physiological performance (Ma et al., 2020; Kuo et al., 2020; Drira et al., 2021). The reaction of ROS with membrane lipids induces lipid peroxidation producing free oxidized fatty acids called oxylipins (Yu et al., 2020). Oxylipins were reported as markers of oxidative stress in plants and microalgae. They alter cell membrane functions or even cause cell death (Yu et al., 2020).

To minimize these damages caused by salt stress, microalgae have developed many physiological, metabolic and molecular mechanisms (Wang et al., 2018). These mechanisms include the accumulation of lipids and carbohydrates as storage molecules to maintain microalgae survival (Anand et al., 2019; Gaubert et al., 2019). Microalgae accumulate ROS-scavenging enzymes such as catalase (CAT), peroxidase (POD), glutathione reductase (GR), ascorbate peroxidase (APX), superoxide dismutase (SOD), and osmoprotectant molecules such as proline, betaine-glycine and carbohydrates etc., which can also act as ROS scavenging molecules (Pancha et al., 2015; Ismaiel et al., 2018; Drira et al., 2021). *C. reinhardtii* is a good model organism for studying cellular salt stress responses in photosynthetic organisms (Raven and Girard-Bascou, 2001). *C. reinhardtii* is relatively flexible and adaptable to changing environmental conditions (Chisti, 2007; Wang et al., 2018; Zuo, 2019). The present study focuses on the physiological, biochemical and metabolomic responses of *C. reinradtii* to salt stress.

2. Material and methods

All chemicals were purchased from Sigma-Aldrich (France).

2.1. Microalga culture conditions and growth

The freshwater green microalga *C. reinhardtii* of the Mascir (Moroccan Foundation for Advanced Science, Innovation and Research) algotech collection was cultivated in 1 *L erlenmeyer* flasks containing 1 L BG11 medium (Stanier et al., 1971) at 25 ± 1 °C. The culture initial concentration was OD_{680} 0.1, aerated using Sherwood Scientific M851 air compressor for uniform mixing (6 L min^{-1} aeration rate), under 81 µmol m^{-2} s^{-1}continuous illumination, using by fluorescent lamps. After one week of microalgae growth at normal conditions, the flasks were separated in control and salt stressed groups. 4 g L^{-1} NaCl was applied to the culture medium every 2 days, for six days. The final NaCl concentration was 200 mM. Each culture group consisted of three replicates. *C. reinhardtii* growth was evaluated twice daily by measuring the optical density (OD) at 680 nm using UV/VIS spectrophotometer brand Ultrospec 3100 pro. The data obtained was converted to log values for the growth curves and the calculation of specific growth rates. The specific growth rate was calculated using the following formula (1):

$$Specific\ growth\ rate\ \mu = ln\,(N2/N1)/(T2-T1) \quad (1)$$

where N1 and N2 are the initial and final OD_{680} at initial time (T1) and final time (T2), respectively.

After 16 days, the biomass was harvested by centrifugation (Centrifuge Heraeus Megafuge 40R) at 2054×g for 10 min, at 4 °C. The harvested biomass was dried in an oven at 50 °C for 48 h and weighed. The biomass productivity was determined as follows (2):

$$Biomass\ productivity\ mg\ L^{-1}\ d^{-1} = \frac{Biomass\ yield\ mg\ L^{-1}}{Number\ of\ days} \quad (2)$$

2.2. Determination of photosynthetic pigments content and biochemical analysis

The photosynthetic pigments of *C. reinhardtii* were extracted and determined at the end of the experiment according to the method described by Xiong et al. (2017). The concentration of pigments was calculated using the formulae ((3, 4, 5) described by (Lichtenthaler, 1987):

$$Chlorophyll\ a\ \left(mg\ L^{-1}\right) = 16.82 \cdot A665 - 9.28\ \cdot\ A652 \quad (3)$$

$$Chlorophyll\ b\ \left(mg\ L^{-1}\right) = 36.92 \cdot A652 - 16.54 \cdot A665 \quad (4)$$

$$Carotenoids\ \left(mg\ L^{-1}\right) = (1000 \cdot A470 - 1.91 \cdot Ca - 95.15 \cdot Cb)/225 \quad (5)$$

Proteins were extracted according to the method described by Fleurence et al. (1995) with a few modifications. 10 mg of the dried microalgae biomass was suspended in 500 µL of deionized water, heated at 90 °C and altered by sonication at 40 kHz every 30 min for 2h30 min. The resultant slurry was then centrifuged at 7715 rpm, 4 °C for 20 min. The supernatant was collected and the pellet was re-suspended in 500 µL sodium hydroxide (0.1 mol L^{-1}) and the method was repeated. The supernatants were combined and the pellet was discarded. The Protein content was determined according to the Bradford (1976) method by recording the absorbance at 595 nm (Spectra Max Plus Molecular Devices spectrophotometer), using bovine serum albumin (BSA) as standard.

The total sugar was determined according to the phenol-sulfuric method with some modifications (Dubois et al., 1956). 10 mg of the dried microalgal biomass was homogenised in 2 mL 2% sulfuric acid with 95–97% purity. The mixture was then heated at 90 °C for 2 h and sonicated for 10 min each 30 min. The hydrolysate was centrifuged and the supernatant was used for the determination of the sugar content as described in the rest of Dubois method. The total sugar content was estimated using glucose as standard, at 490 nm.

Total lipids were extracted according to the method described by Bligh and Dyer (1959), with slight modifications. 100 mg of the dried microalgal biomass was added by the following proportions Water/Methanol (99.8%)/Chloroform (99–99.4%): 1/1/2 (v/v/v). The mixture was sonicated for 15 min and centrifuged 4500 rpm and 4 °C for 10 min. The organic phase was recuperated and the lipids washed with NaCl 0.9% in a separating funnel. The solvent was evaporated using nitrogen gas and the lipids were weighted and stored at -20 °C. Fatty acid methyl esters (FAME) and metabolites were determined after transesterification as described in the previous work (Fal et al., 2021). Total phenols were colorimetrically determined using Folin-Ciocalteu reagent as described by Velioglu et al. (1998) with slight modifications. 200 mg of fresh microalgal biomass was suspended in 2 ml of 80 % ethanol, sonicated for 60 min at 40 KHz and centrifuged at 3396 ×g for 10 min at 4 °C. The supernatant was recuperated in another tube wrapped in aluminium foil. The pellet was recovered and the extraction method was repeated. The two extracts were mixed and stored at -20 °C for analysis. 200 µl of the extract was added to 100 µl Folin-Ciocateu reagent (diluted tenfold with DW) and incubated in darkness at room temperature for 5 min. Then, 800 µl of sodium carbonate (75 mg L^{-1}) was added to the mixture and incubated for 60 min in darkness at room temperature. The absorbance was measured at 765 nm and the total phenolics were quantified using gallic acid as standard. The phenols content expressed in g L^{-1} as gallic acid equivalent (GAE).

2.3. Measurement of oxidative and antioxidant activities

2.3.1. Determination of stress biomarkers H_2O_2, MDA and proline content

The H_2O_2content was determined according to the method described by Velikova et al. (2000) with a few modifications. 0.1 g of the fresh

microalgal biomass was homogenized in 1 mL of 0.1% (w/v) trichloroacetic acid (TCA) on ice and centrifuged at 12, 000 ×g for 15 min. 0.5 ml of the supernatant was mixed with 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1 mM KI. H_2O_2 content was calculated using a standard curve, at 390 nm absorbance.

For proline and malondialdehyde (MDA) determination, the extraction was done by homogenizing 0.1 g of the microalgal biomass in 1.5 ml 100 mM PBS buffer (pH 7.8) and centrifuged at 4 °C, 10,000 × *g* for 20 min. For proline content determination, the method described by Bates (1973) was used with slight modifications. 0.1 ml of crude protein was mixed in 0.9 mL 3% sulphosalicylic, acetic acid and 2.5% acid-ninhydrin. The reaction mixture was heated at 95 °C for 15 min. The reaction was cool down on ice for 5 min. The proline content was determined at 520 nm absorbance using L-proline as standard (Spectra max Plus molecular devices spectrophotometer). The MDA content was determined according to the Heath & Packer method with slight modifications (Heath &Packer, 1968). 0.1 ml of the crude protein was mixed in 0.25 % TBA, 10% of TCA and incubated at 95 °C for 15 min. The reaction was stopped by placing the reaction mixture on ice for 5 min. The MDA content was derived from the difference in absorbance at 532 nm and 600 nm, using the extinction coefficient 156 mM^{-1} cm^{-1}, and calculated using the following formula (6):

$$MDA\,(nmol/mgprotein) = (A532 - A600) \times Vr \times (V/Vt)/155 \times 1{,}000/Cp \quad (6)$$

Where: A532 is the absorbance at 532 nm, A600 the absorbance at 600 nm, Vr the volume of reaction mixture, V the total volume of crude protein solution, Vt the volume of crude protein used in the testing tube, Cp: crude protein concentration (mg mL^{-1}) and 155 is the MDA-TBA extinction coefficient at 532 (mM^{-1} cm^{-1}).

2.3.2. Scavenging enzymes assays

The enzyme extract was prepared by homogenizing 0.1 g fresh microalgal biomass in 1 mL extraction buffer containing 100 mM sodium phosphate buffer (pH 7.8), 0.1 mM EDTA, 1% (w/v) polyvinyl pyrrolidone (PVP) and 0.5% (v/v) triton X-100. The crude protein concentration in the supernatant was estimated according to the Bradford method using BSA as standard for protein quantification (Bradford, 1976).

CAT activity was determined according to Aebi (1974) with slight modifications. 0.1 ml of the enzyme extract was mixed with 50 mM phosphate buffer (pH 7.0) and 20 mM H_2O_2. Decrease in absorbance was measured for 3 min at 240 nm. One unit of CAT activity is defined as the amount of enzyme required to oxide 1 mM of H_2O_2 per min.

The enzyme activity was calculated according to the following formula (7):

$$CAT\ activity\ (unit\ /\ mg\ protein) = \Delta A240 \times (V/Vt)/(0.1 \times t)/Cp \quad (7)$$

Where △A240 equals the change in absorbance at 240 nm every 15 sec, V equals the total volume of the crude enzyme solution, Vt equals the volume of the crude enzyme used in the testing tube, t equals the reaction time (min), Cp is the crude protein concentration (mg mL^{-1}) and 0.1 is the decreased absorbance at 240 nm by one unit of CAT.

POD activity was determined according to the guaiacol oxidation method (Kar and Mishra, 1976). The POD activity was assayed by adding 0.1 mL enzyme solution to 0.9 mL of the reaction mixture containing 28 µl 0.2 % guaiacol and 19 µl 30 % H_2O_2. The final volume was completed to 50 ml by adding 100 mM PBS (pH 7.0). The absorbance was recorded at 470 nm every 15 sec during 3 min. One unit of enzyme activity was equal to OD470 increase 0.01 per min.

The enzyme activity was calculated using the following Eq. (8):

$$POD\ activity\ (Unit\ /\ mg\ protein) = (\Delta Abs \times Vt)\ /\ (\Delta t \times \varepsilon \times l \times v)/\ Cp \quad (8)$$

Where Δt is the time of incubation (min), Δ Abs is the change in absorbance, Vt is the total assay volume, V is the enzyme sample volume, ε is the extinction coefficient of substrates in units of M^{-1} cm^{-1}, l is the cuvette diameter (1 cm) and Cp is the crude protein content.

The enzyme activity (Unit) was defined as the amount of enzymes that oxidized 1µmol of substrate/min.

SOD activity was determined according to the Beauchamp and Fridovich (1971) method with slight modifications. The enzyme extract (0.1 mL) was added to 0.9 mL reaction mixture containing 100 mM phosphate buffer (pH 7.8), 130 mM L-methionine, 1mM $EDTA_2Na$, 20 µM riboflavin and 750 µM nitro blue tetrazolium (NBT). 100 µL reaction solution containing 100 mM PBS (pH 7.8) without the crude enzyme under dark and light conditions (4000 lux) served as controls I and II, respectively. The change in absorbance was recorded at 560 nm. One unit of SOD activity was measured as 50% reduction in NBT. The enzyme activity was calculated according to the following formula (9):

$$SOD\ \ total\ \ activity\,(unit:u/mgprotein) = [(Ack - As) \times V]/(0.5 \times Ack \times Vt)/Cp \quad (9)$$

Where: Ack is the absorbance at 560 nm of Control II (exposed to light with no crude enzyme), As is the absorbance at 560 nm of candidate sample tube, V is the total volume of the crude enzyme solution, Vt is the crude enzyme volume used in the testing tube, Cp is the crude protein concentration (mg mL^{-1}) and 0.5: One unit of SOD is defined as the amount of enzyme that inhibits 50% nitroblue tetrazolium photoreduction.

For the APX assay, 0.1 g biomass was homogenized in 50 mM PBS buffer (pH 7.0) containing 0.2 mM PMSF and 0.35 mM ascorbate to prevent inactivation of the enzyme. The homogenates were centrifuged at 10,000 ×g for 15 min at 4 °C and stored at −20 °C for further use. APX activity was determined according to the method described by Nakano and Asada (1981) with slight modifications. 10 µg crude protein was mixed in 1 ml of the reaction mixture containing 50 mM of sodium phosphate buffer (pH 7.0), 0.35 mM of Ascorbic acid and 10 mM of H_2O_2. The hydrogen peroxide-dependent oxidation of ascorbate was determined by recording the decrease in absorbance at 290 nm every 15 sec for 3 min, using an absorption coefficient 2.8 mM^{-1} cm^{-1}. The enzyme activity was calculated using the following formula (10):

$$POX\ (Unit\ /\ mg\ protein) = ((\Delta Abs \times Vt)\ /\ (\Delta t \times \varepsilon \times l \times V))\ /\ Cp \quad (10)$$

Where Δt is the time of incubation (min), ΔAbs is the change in absorbance, Vt is the total assay volume, V is the enzyme sample volume, ε is the extinction coefficient of substrates (M^{-1} cm^{-1}), and l is the cuvette diameter (1 cm). The enzyme activity (Unit) was defined as the amount of enzymes that oxidized 1µmol of substrate/min. Cp is the crude protein concentration mg L^{-1}.

2.4. Statistical analysis

Statistical analyses and graphs were performed using GraphPad perism 9 software. Descriptive statistics and significant differences of the mean values were determined using Student's t-test at $p \leq 0.05$. Correlation was performed with R studio using correlation packages. All experiments in the work were performed in three replicates; the results were represented as arithmetic mean ± standard deviation.

3. Results and discussion

3.1. The effect of salinity stress on microalgal growth, photosynthetic pigment content and biomass productivity

C. reinhardtii was exposed to 0 and 200 mM NaCl during eight days at exponential phase to investigate the effect of salt stress on growth, biomass productivity and photosynthetic pigments (Figure 1, Figure 2).

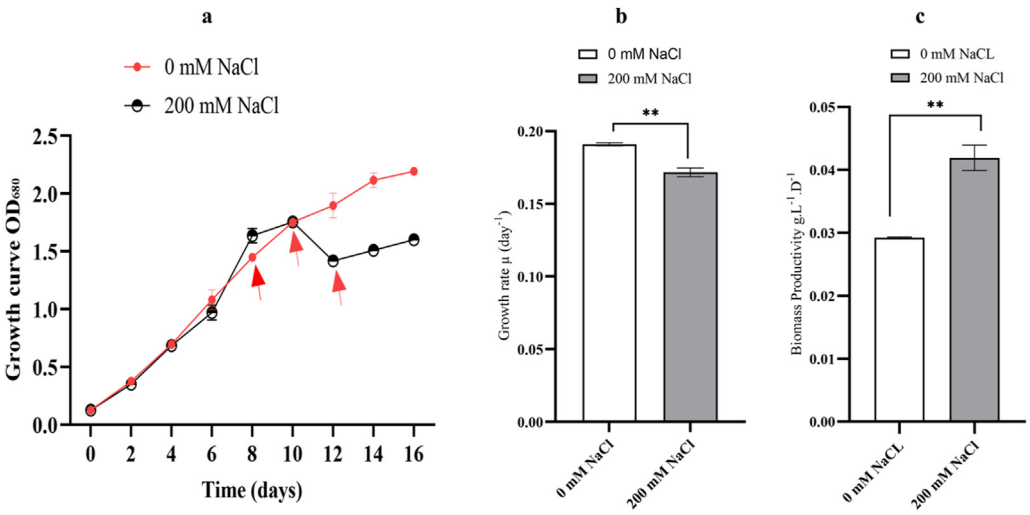

Figure 1. Effect of salinity on *C. reinhardtii* growth curve (a) the arrow indicates the day of salt addition., growth rate (b), biomass productivity (c) Data are presented as means and standard errors of three repetitions. White bars 0 mM NaCl and Grey bars 200 mM NaCl. Asterisks indicate a statistically significant difference **p ≤ 0.01 according to Student's t-test.

C. reinhardtii growth rate was assessed through optical density (OD_{680} nm). During the 8 days of exposure to 200 mM NaCl, the specific growth rate of *C. reinhardtii* was significantly reduced from 0.19 ± 0.001 day^{-1} to 0.17 ± 0.003 $day^{-1,}$ respectively. The biomass productivity was significantly increased from 0.029 ± 0.000 g L^{-1} d^{-1} to 0.042 ± 0.002 g L^{-1} d^{-1} at 0 and 200 mM, respectively. NaCl in low concentration is beneficial for some metabolic functions and increase microalgae growth. However, high concentrations might inhibit growth and cause cell death (Pandit et al., 2017; Rezayian et al., 2019; Qiao et al., 2021). Numerous studies reported that growth rate is reduced with increasing of NaCl concentration (Ji et al., 2018a; Singh et al., 2018). Concominatly, Hounslow et al., (2021) recorded a reduction in the growth rate of *C. reinhardtii* from 1.93 × 10^6 cells day^{-1} to 1.08 × 10^6 cells day^{-1} subjected to 200 mM NaCl. The growth of *Chlorella sp. TLD6B* was reduced from OD_{680} 1.3 to 1 after the application of 200 mM NaCl with significant increase in biomass productivity from 0.022 g L^{-1} d^{-1} to 0.0277 g L^{-1} d^{-1} (Li et al., 2021). The salt stress significantly affected the growth of *C. reinhardtii,* notably in the range 25–200 mM NaCl where the treatments caused a decrease in cell density after gradually suppressed cell growth (Fan and Zheng, 2017). Fan and Zheng (2017) also reported that *C. reinhardtii* subjected to 200 mM NaCl led to permanent cell damage of decreased cell growth, with 47% cell death 3 days after treatment. Cell death results when the dynamic balance between ROS consumption and production is destroyed by the excess of salt ions (Ji et al., 2018a). Alteration of osmotic potential and membrane permeability was another reason for growth inhibition causing changes in cellular ionic ratios due to the membrane selective ion permeability, ion transport, water potential and solubility of CO_2 and O_2 (Singh et al., 2018). Microalgae also showed significant accumulation of biomass in spite of the growth rate inhibition. This may be due to the size of the cell and organelles (chloroplast, mitochondria, vacuole etc.) which are bigger and heavy under salinity stress compared to the control (Sinetova et al., 2021). In addition, the accumulation of osmoprotectant molecules such as proteins, glycerol, proline etc. balance the osmotic pressure induced by sodium chloride and prevents water loss (Anand et al., 2019). In the present study, there was a positive correlation between biomass productivity and osmoprotectant molecules (carbohydrates, proline and polyphenols) as shown in Figure 5. Salt toxicity targets chlorophyll (Chl) causing Chl and growth reduction by limiting net CO_2 assimilation rate (Kirrolia et al., 2011; Pandit et al., 2017; Hounslow et al., 2021). In this context, photosynthetic pigments, Chl a, Chl b and carotenoids were measured at the end of the experiment (Figure 2). Chl a and b of *C. reinhardtii* were reduced significantly from 7.15 ± 0.372 mg L^{-1} and 1.67 ± 0.360 mg L^{-1} to 5.28 ± 0.477 mg L^{-1} and 1.24 ± 0.243 mg L^{-1} at 0 and 200 mM NaCl, respectively (Figure 2). The reduction of Chl a may be an oxidative stress symptom related to increased chlorophyllase activity promoting Chl a degradation (Ji et al., 2018). It may also be related to decrease in Rubisco activity due to the low CO_2 uptake (Rezayian et al., 2019; Hounslow et al., 2021). It's reported that salt stress negatively affects carbon fixation and carbon concentrating mechanisms in *C. reinhardtii* which is required for CO_2 availability for Rubisco (Hounslow et al., 2021). Pandit et al. (2017) related this chlorophyll reduction to osmotic and toxic ionic stress, which causes a decrease in photosynthetic rate and consequently lowers Chl and protein content. In addition, the concentration of Chl a+b in *C. reinhardtii* was remarkably decreased from 14 μg mL^{-1} at 0 mM NaCl to 9 μg mL^{-1} under 200 mM NaCl conditions (Hang et al., 2020). Conversely, cyanobacteria and

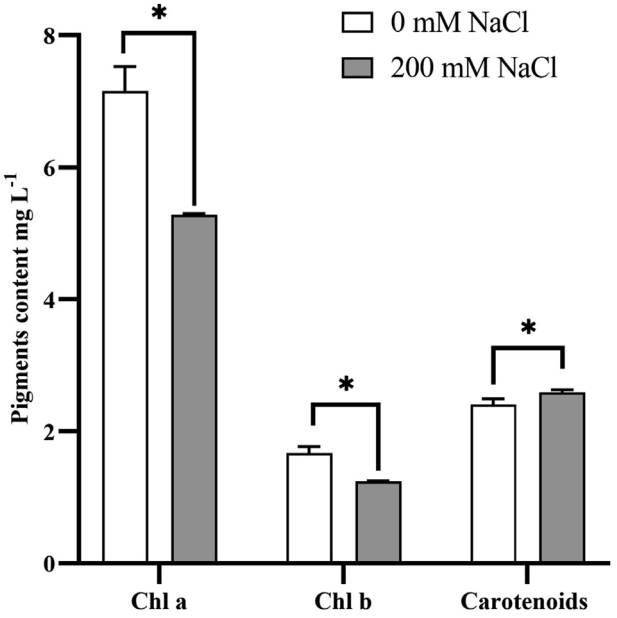

Figure 2. Effect of salinity on photosynthetic pigments Chl a, Chl b and carotenoids of *C. reinhardtii*. Data are presented as means and standard errors of three repetitions. White bars 0 mM NaCl and Grey bars 200 mM NaCl. Asterisks indicate a statistically significant difference *p ≤ 0.05 according to Student's t-test.

microalgae accumulate carotenoids under unfavourable conditions as antioxidative molecules against oxidative damage and lipid peroxidation (Ji et al., 2018b; Rezayian et al., 2019). In the present study, the carotenoids in NaCl-treated was 2.60 ± 0.242 mg L^{-1} compared to the control with 2.40 ± 0.119 mg L^{-1} Rezayian et al. (2019) reported that carotenoids play an important role in photosynthesis and plant development as well as stress responses.

3.2. ROS content and response of antioxidant system of C. reinhardtii to salinity stress

In the present study, the effect of salt stress on ROS generation was investigated by measuring H_2O_2 (Figure 3a). The results showed a significant ($P \leq 0.01$) increase in H_2O_2 under 200 mM NaCl conditions with 10.14 ± 1.126 mM compared to 4.88 ± 0.665 mM in the control. Yun et al. (2019) reported that *Chlorella vulgaris* (*C. vulgaris*) accumulated 0.2028 mM 0.1g^{-1} FW (Fresh weight) under 200 mM NaCl stress conditions compared to 0.0347 mM 0.1 g^{-1} FW. H_2O_2 is a reactive oxygen species (ROS) that plays a role in the control of plant and algae responses to various stressors (Qiao et al., 2021). H_2O_2 production is indispensable for abscisic acid (ABA) and brassinosteroids (BRs) that enhance stress tolerance in plants and algae (Qiao et al., 2021). On the other hand, ROS formation may induce change in microalgae metabolism such as the reduction of nutrient uptake and CO_2 flux with increased nicotinamide adenine dinucleotide phosphate (NADPH) formation (Srivastava et al., 2017). Moreover, ROS induce photoreduction, thylakoid membrane lipids peroxidation and triplet Chl formation (Srivastava et al., 2017; Ji et al., 2018a; Singh et al., 2018). Lipid peroxidation is another stress biomarker widely used in microalgae measured in terms of MDA content in the cells (Pancha et al., 2015; Wang et al., 2020). The peroxidation of membrane lipids is the main indicator of cell damage (Acet and Kadıoğlu, 2020; Xudong et al., 2020). MDA is the main product of lipid peroxidation generated from the oxidation of polyunsaturated fatty acid by free radicals. MDA is considered as stress oxidative marker (Meng et al., 2020; Yang and Hu, 2020). In parallel with the H_2O_2 results, MDA content in the NaCl-treated cultures was 62.88 ± 4.428 nmol mg^{-1} of protein compared to the control (24.04 ± 0.329 nmol mg^{-1} of protein) as shown in Figure 3b. To further understand ROS-mediated lipid accumulation in *Auxenochlorella protothecoides*, Polat et al. (2020) measured H_2O_2 and MDA levels. Their study indicated an increase (3.9-fold) in H_2O_2 from 5.60 μmol g^{-1} to 22.33 μmol g^{-1} and a 4.4 fold increase in MDA, from 0.13 nmol g^{-1} to 0.57 nmol g^{-1} at 40 g L^{-1} NaCl. In addition, MDA levels in *Micractinium sp.* increased sharply from 0.2 μmol g^{-1} to 0.57 μmol g^{-1} at 40 g L^{-1} after 3 days (Yang and Hu, 2020). Thus, the increase in MDA level was related to oxidative stress resulting from the exposure to excessive NaCl concentration in medium (Polat et al., 2020). Many studies have reported the accumulation of MDA in plant and microalgae cells as a product of lipid peroxidation under salt stress (Yang and Hu, 2020; Zheng et al., 2020; Qiao et al., 2021).

Microalgae have a defence system involving osmoprotectants, non-enzymatic molecules and antioxidant enzymes which are significantly activated under stress conditions (Kumar et al., 2020; Wang et al., 2020). In this study, proline and polyphenols content as non-enzymatic scavengers and SOD, POD, CAT and APX as enzymatic antioxidants were analysed. Proline is an important amino acid that has multiple functions in response to stress, acting as an osmolyte, ROS scavenger and a molecular chaperone stabilizing the structure of proteins. It is the most extensively accumulated molecule as a stress indicator to protect cells from ROS damage (Tietel et al., 2019; Zalutskaya et al., 2020). In the present study *C. reinhardtii* showed a highly significant accumulation of proline under salt stress (225.17 ± 3.910 μg mg^{-1} proteins) compared to 69.55 ± 0.264 μg mg^{-1} protein in the control (Figure 3c). Previous studies have reported, increased proline levels under salt stress conditions (Rezayian et al., 2019; Shahid et al., 2020). In addition, *Nostoc piscinale* displayed a significant accumulation of proline under 200 mM NaCl conditions by 11 μg g^{-1} (FW) compared to 5 μg g^{-1} (FW) 0 mM NaCl conditions (Rezayian et al., 2019). In addition, Chokshi et al. (2017) showed that exposure of *Acutodesmus dimorphus* to 200 mM NaCl induced proline accumulation by 1.8 fold (66.45 ± 1.07 μM g^{-1} DW compared to control 37.36 μM g^{-1} DW). Enhanced synthesis, lowers the rate of oxidation, and slow incorporation into proteins were the main cause of proline accumulation under stress conditions (Rezayian et al., 2019). Polyphenols are powerful ROS scavengers that increase cell membrane integrity and protect cells from oxidative damage (Chokshi et al., 2017). Like proline, they act as antioxidant molecules helping in osmotic adjustment and scavenging free radicals to reduce the negative effects of salt stress (Kumar et al., 2020; Drira et al., 2021). In the present study, *C. reinhardtii* showed a significant accumulation of polyphenols under NaCl stress conditions (0.53 ± 0.037 g L^{-1}) compared to 0.35 ± 0.017 g L^{-1} in the control ($p < 0.01$) (Figure 3d). In accordance, a previous study showed significant accumulation of polyphenols in *Acutodesmus dimorphus* under 200 mM NaCl conditions 149.24 μg g^{-1} FW compared to 138.49 μg g^{-1} FW in the control after 3 days of culture (Chokshi et al., 2017). SOD act as the first line of defense against ROS, these enzymes protect cells from oxidative stress by the dismutation of superoxide radical to molecular oxygen and H_2O_2 (Singh et al., 2018; Moghimifam et al., 2020; Zhang et al., 2020). In the present study, the activity of SOD decreased significantly under 200 mM NaCl conditions. The SOD activity

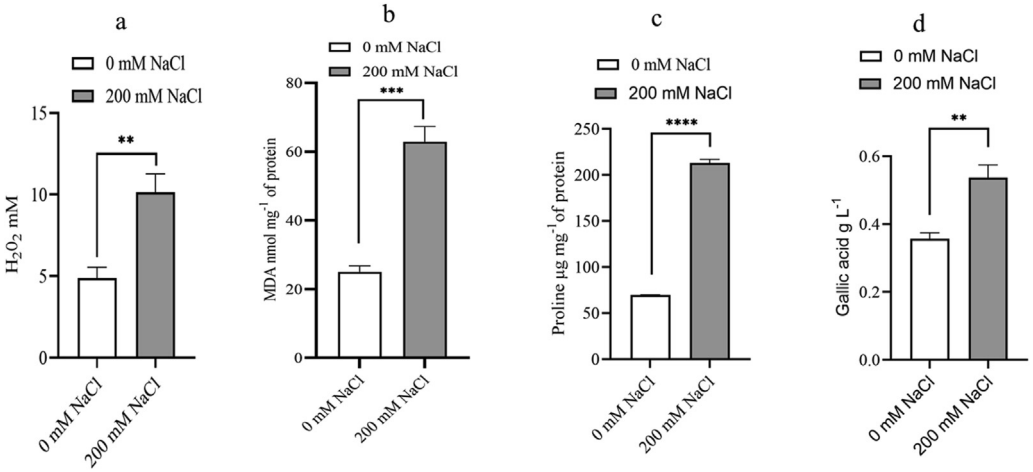

Figure 3. Accumulation of H_2O_2 (a), MDA (b), proline (c) and polyphenols expressed by gallic acid (d) under salinity stress. Data are presented as means and standard errors of three repetitions. White bars 0 mM NaCl and Grey bars 200 mM NaCl. Asterisks indicate a statistically significant difference **$p \leq 0.01$; ***$p \leq 0.001$; ****$p < 0.001$ according to Student's t-test.

in the NaCl-treated culture was 2.59 ± 0.696 U mg^{-1} of protein compared to the control (8.36 ± 0.565 U mg^{-1} of protein) as shown in Figure 4a. The decrease in SOD activity may hamper the cells O_2^- scavenging mechanism and promote the accumulation of O_2^- and H_2O_2, lowering SOD activity (Farghl et al., 2015). This indicates that SOD intervened in the microalgal stress response due to the increased H_2O_2 levels (10.14 ± 1.126 mM) under 200 mM NaCl conditions, compared to 4.88 ± 0.665 mM in control. In a study by Zhang et al. (2018), SOD activity first increased after 1 h, peaked at 12h and then gradually decreased under salt stress. H_2O_2 acts as a signalling molecule in environmental stresses and adaptive responses, but is also harmful to cellular membranes (Hu et al., 2012; Moghimifam et al., 2020). Furthermore, H_2O_2 was dismutated to H_2O and O_2 by catalase and into two molecules of water by peroxidase and ascorbate peroxidase using ascorbic acid as the electron donor (Singh et al., 2018; Wang et al., 2020; Zhang et al., 2020). As a result, the enzymes CAT, SOD, and POD form a full antioxidant chain (Chokshi et al., 2017). Our results showed a significant increase in POD and CAT levels under salt stress conditions, which were 12.15 ± 0.125 U mg^{-1} of protein and 2.07 ± 0.093 U mg^{-1} of protein, respectively compared to 3.11 ± 0.336 U mg^{-1} of protein and 1.64 ± 0.149 U mg^{-1} of protein in the control, respectively (Figure 4b, c). However, a decrease in APX level was noted under 200 mM NaCl, which was 0.020 ± 0.005 U mg^{-1} of protein compared to the control with 0.027 ± 0.003 U mg^{-1} of protein (Figure 4d). In response to oxidative stress, the activity of these enzymes change, indicating the presence of an antioxidative mechanism in the cells (Chokshi et al., 2017). The decrease in SOD and APX activities and increase in CAT and POD was in accordance the results obtained by Chokshi et al. (2017) where SOD and APX activities in *Acutodesmus dimorphus* significantly reduced under 200 Mm NaCl conditions after 2 days. SOD and APX activities were 835.88 U mg^{-1} of protein and 11.75 U mg^{-1} of protein under salt stress compared to 1308.11 U mg^{-1} of protein and 18.85 U mg^{-1} of protein in normal conditions, respectively. In the same study, there was a significant increase in CAT activity after 3 days (15.87 U mg^{-1} protein) under 200 mM NaCl conditions, compared to 14.35 U mg^{-1} protein (Chokshi et al., 2017). These findings indicate that changes in antioxidant enzyme activities and low-molecular compounds depend on the microalgal species, development stage and metabolic state, as well as the period of exposure to stress and salt concentration applied (Hu et al., 2012). In response to salinity stress *C. reinhardtii* induced the antioxidant system to scavenge ROS and Figure 5 shows a correlation between ROS and scavenging enzymes and molecules. Among non-enzymatic antioxidants, proline and polyphenols highly accumulated in *C. reinhardtii* and positively correlated with ROS (H_2O_2). Considering enzymatic antioxidants, *C. reinhardtii* showed increased POD and CAT activities under 200 mM NaCl conditions, which reduces the high levels of H_2O_2 induced by salt stress. They also have a positive correlation with H_2O_2.

3.3. Biochemical composition and metabolomic changes in C. reinhardtii under salinity stress

The content of proteins, lipids and carbohydrates under 0 and 200 mM NaCl conditions was measured. The results presented in Figure 6 showed that salt stress induces a significant decrease in protein content from 14.33% to 8.50% under 0 and 200 mM NaCl conditions, respectively. There was also a significant increase in lipids and carbohydrates content which increased from 9.35% and 27.18%–14.3% and 46.78% under 0 and 200 mM NaCl conditions, respectively. Several studies reported that unfavourable conditions such nutrient limitation or high salinity stimulate higher lipid and carbohydrate accumulation in microalgae compared to proteins (Shen et al., 2015; Mirizadeh et al., 2020; Hang et al., 2020). Previous studies reported that microalgae accumulate carbohydrates in the first stage of stress as a quick response to stress and switch to lipid synthesis by metabolic pathways (Siaut et al., 2011; Hang et al., 2020; Hounslow et al., 2021). Microalgae use carbohydrates in salt stress as osmoprotectants to regulate homeostasis and assure osmotic adjustment (Wang et al., 2018; Tietel et al., 2019). In addition, carbohydrates content helps cells to adapt to salt stress (Pandit et al., 2017). In the present study, subjecting *C. reinhardtii* to NaCl stress for 8 days, significantly increased the accumulation of carbohydrates by 46.78% compared to the control (27.18%). Sugar content in *C. reinhardtii* increased from 3% to 5% under salt stress (200 mM NaCl for 80 h) (Hounslow et al., 2021). Accumulation of carbohydrates in response to salt stress have been previously reported in many microalgae strains involving *Chlamydomonas* sp., *Chlorella* sp., *Dunaliella* sp., *Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp. (Arora et al., 2019). Salt stress induces lipid accumulation in microalgae (Anand et al., 2019). High NaCl concentration (200 mM) significantly increased lipid content (41.1%) in *C. reinhardtii* compared to the control (20.8%) after 3 days of application (Hang et al., 2020). *C. reinhardtii* also accumulated lipids (0.348 and 0.437 g g^{-1} dry biomass) under 0 and 150 mM NaCl conditions, respectively (Ji et al., 2018). This accumulation of lipids, specifically neutral lipids, play a role in the maintenance of the membrane integrity in response to salt stress, causing decrease in cell membrane osmotic pressure and fluidity (Ji et al., 2018a). This study investigated metabolic changes in *C. reinhradtii* and their function in salt stress. Table 1 shows that *C. reinhradtii* exposure to 200 mM NaCl resulted in increased FAME and unsaturated fatty acids (UFA) content (84.81%

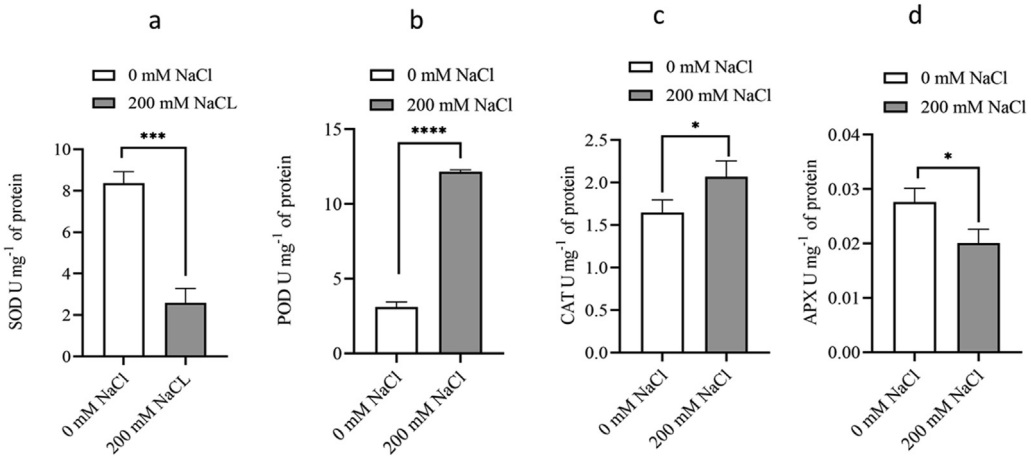

Figure 4. Responses of antioxidative enzymes in *C. reinhardtii* to salinity stress SOD (a), POD (b), APX (c) and CAT (d). Data are presented as means and standard errors of three repetitions. White bars 0 mM NaCl and Grey bars 200 mM NaCl. Asterisks indicate a statistically significant difference *p ≤ 0.05; ***p ≤ 0.001; ****p < 0.001 according to Student's t-test.

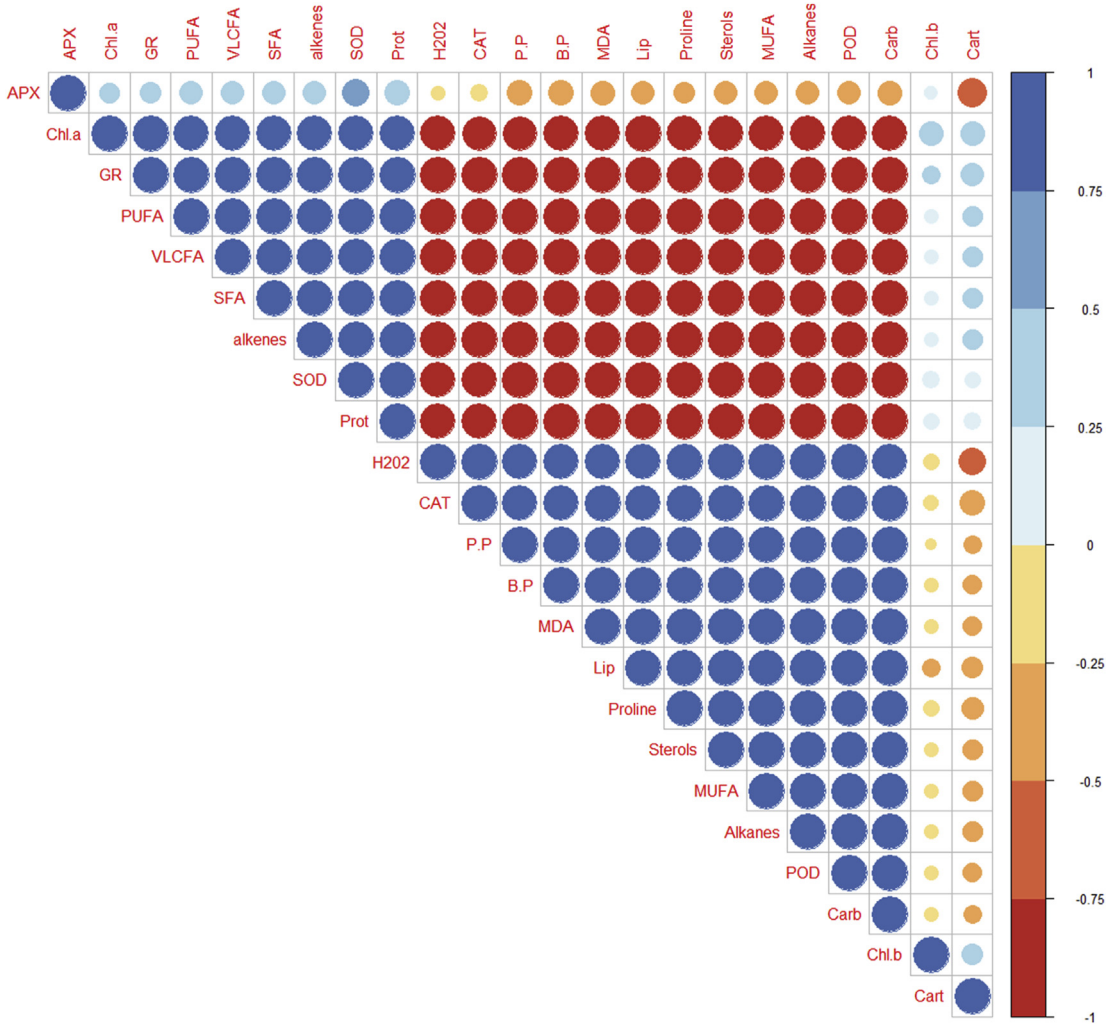

Figure 5. Correlation between parameters measured to assess physiological, biochemical and metabolomic responses of *C. reinhardtii* to salt stress. G.R: growth rate, B.P: Biomass productivity, Chl a, Chl b, Carot: Carotenoids, Prot; Proteins, Lip: Lipids, Carb: Carbohydrates, H_2O_2, MDA, Proline, P.P: polyphenols, SOD, POD, APX, CAT, SFA, VLCFA, MUFA, PUFA, Alkanes, Alkenes and Sterols.

and 60.05%) compared to the control (65.33% and 45.42%) respectively. Conversely, saturated fatty acids (SFA) content decreased by 19.49% under 200 mM NaCl conditions compared to the control (19.91%). This increase in UFA can be considered as the microalgal's response to preserve the membrane from salt alteration. Generally, salt stress causes membrane degradation, resulting in the alteration of membrane permeability, integrity, fluidity and ion transport selectivity (Gogna et al., 2021). Several studies reported the crucial role of UFA in adaptation and tolerance responses to salt stress by protecting the plasma membrane and the photosynthetic machinery (Rismani and Shariati, 2017). Polyunsaturated fatty acids (PUFA) and SFA act as membrane monomers, particularly in the membranes of organelles. Previous studies revealed that decreased PUFA and long chain SFA partitioning inhibits the cell's ability to produce new organelles, thereby limiting cell proliferation (Miquel and Browse, 1995; Jump, 2002; Wan Afifudeen et al., 2021). In the present study, very long chain fatty acid (VLCFA) and PUFA percentage was reduced under salt stress (3.74% and 24.56% compared to the control (3.82% and 39.5%), respectively.

It is important to note that oleic acid content increased by 40.29% under 200 mM NaCl conditions compared to the control (5.92%). No linoleic acid (C18:2) was detected in the culture group subjected to 200 mM NaCl conditions. However, there was an increase in α-linolenic acid (C18:3) for the culture group subjected to 200 mM NaCl (19.29%) compared to the control (17.12%). markedly increased the monounsaturated fatty acids (MUFA), notably C18:1, increased by 30.74% in *Monoraphidium sp.QLY-1* subjected to 171.12 mM NaCl compared with 24.99% in the control (Qiao et al., 2021). The same study also reported a decrease in SFA, notably palmitic acid (33%) under salt stress compared with the control (37%) (Qiao et al., 2021). UFA levels in *Synechococcus elongatus UTEX 2973* also increased by 60.05% under 200 mM NaCl compared with the control (45.42%) (Cui et al., 2020). These findings suggest that salt stress may induce an increase in UFA enzymes (Rismani and Shariati, 2017). Furthermore, Gogna et al. (2021) related the accumulation of oleic acid and decrease in linoleic acid under saline conditions to Δ^{12} desaturase (oleate desaturase) inhibition by the toxic ions such Na^+ and Cl^- that are harmful for lipid metabolism. Δ^{12} desaturase is the key enzyme responsible for linoleic acid synthesis by adding a double bond to oleic acid at position 12 (Ghassemi-Golezani and Farhangi-Abriz, 2018; Gogna et al., 2021). This inhibition may also be due to the restriction of fatty acid transport among organelle. Generally, oleic acid formation occurs in plastids, while desaturation takes place in the cytosol. Thus, salinity stress limits the transport of oleic acid to the cytosol, resulting in high accumulation of oleic acid and decrease in linoleic and linolenic acid content (Flagella et al., 2004; Ghassemi-Golezani and Farhangi-Abriz, 2018).

In this study, salt stress induced a reduction in palmitic acid and stearic acid (13.33% and 1.88%) respectively compared with the control (13.74% and 2.14%), respectively. However, there was an increase in the

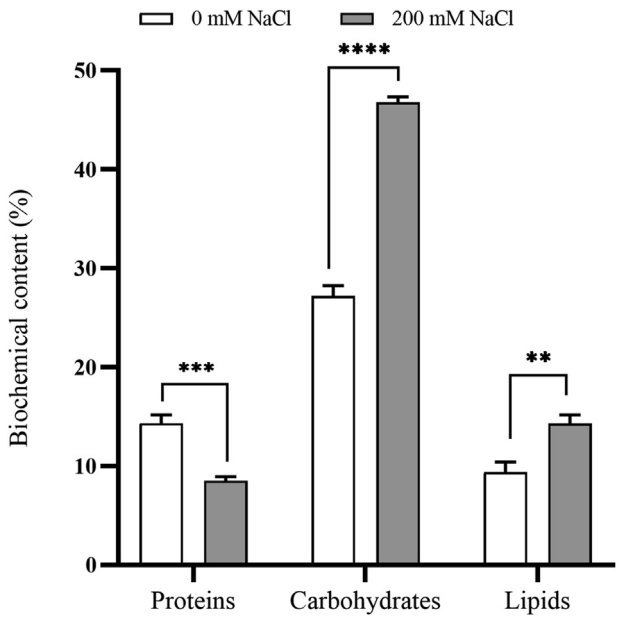

Figure 6. Effect of salinity on the biochemical composition (proteins, carbohydrates and lipids content) of *C. reinhardtii*. Data are presented as means and standard errors of three repetitions. White bars 0 mM NaCl and Grey bars 200 mM NaCl. Asterisks indicate a statistically significant difference **p ≤ 0.01; ***p ≤ 0.001; ****p < 0.001 according to Student's t-test.

level of oleic acid C18:1 and linolenic acid C18:3. Oleic acid serves as the main product of *De novo* fatty acid synthesis which produces omega 3 such linolenic acid C18:3, eicosapentaenoic acid C20:5 and etc. This class of FA play a major role in biotic and abiotic stress responses (Wen and Chen, 2003; Solovchenko, 2012; Chen et al., 2019). Oleic acid and palmitic acid produced from aerobic desaturation and chain elongation act as precursors of membrane glycerolipids (Hempel et al., 2012). Moreover, oleic acid takes the acyl editing pathway rather than the multistep of de novo synthesis pathway to be directly incorporated into phosphatidylcholine (PC) as a vector of the desaturation for membrane glycerolipids biogenesis (He and Ding, 2020).

Table 1. The effect of salinity stress on the distribution of FAME (Area %) in *C. reinhardtii*.

	NaCl 0 mM	Nacl 200 mM
C14:0	0.21	0.18
C15:0	-	0.04
C16:0	13.74	13.33
C18:0	2.14	1.88
C20:0	-	0.32
C22:0	0.58	0.53
C24:0	0.75	0.6
C26:0	0.48	0.37
C28:0	2.01	2.14
C30:0	-	0.1
SFA	19.91	19.49
VLCFA	3.82	3.74
C18:1	5.92	40.29
C20:1	-	0.47
MUFA	5.92	40.76
C18:2	22.38	-
C18:3	17.12	19.29
PUFA	39.5	19.29
Unknown FA	-	5.27
Total	65.33	84.81

The accumulation of fatty acids in microalgae under stress is related to growth stage (Teh et al., 2021). Teh et al. (2021) reported that salt stress increased C18:1 and C18:2 levels in *C. vulgaris* at mid and late-stationary phases. C18:1 and C18:2 can be readily converted into C18:3n3 by ω -3/ω -6 FAD enzymes which is a critical modulator of growth and stress response. Generally, linolenic acid C18:3 acts as a precursor of the oxylipin pathway involving jasmonic acid (JA) (Sembdne and Parthier, 1993; Wasternack, 2007; Gogna et al., 2021). As a plant growth regulator, jasmonate has a role in algal growth and development, including protein synthesis, fatty acid metabolism, and stress resistance (Christov et al., 2001). Exogenously application of JA-Me (methyl jasmonate) 100 μM (1. 10^{-4}) enhanced the tolerance of *Scenedesmus incrassatulus* to the toxic salinity effects under 175 mM NaCl conditions (Fedina and Benderliev, 2000). Several studies related the accumulation of linolenic acid (18:3) to the activation of NADPH oxidase, which are prominent ROS generators in plants and microalgae (Yaeno et al., 2004; Di Palma et al., 2020; Rachidi et al., 2021). Hence, increased ROS (H_2O_2) accumulation in the present study may be due to the activation of NADPH oxidase by the accumulation of linolenic acid, which was increased (19.29%) under 200 mM NaCl conditions, compared to 17.12% in the control.

In this study there was a slight decrease (3.74%) in VLCFA content under 200 mM NaCl conditions, compared to with the control (3.82%). Other molecules such as eicosanoic acid (C20:0), eicosenoic acid or gondoic acid (C20:1) and melissic acid (C30:0) were also detected under 200 mM NaCl conditions at 0.32%, 0.47%, 0.1%, respectively but were absent in the control. These findings contradict with the study by Kondo et al. (2016) which reported that microalga *C. reinhardtii* cannot produce VLCFA and it's cell wall's lipid layer is primarily made up of triacylglycerides (TAG) and alkanes embedded in glycoprotein frameworks. In general, microalgae respond to salt stress by raising VLCFA content as an adaptive response, resulting in membrane rigidification and decreased salt permeability (Wang et al., 2015; Kondo et al., 2016). This class of fatty acid have been identified as the primary precursors in cutin and wax elongation processes (Rashidi and Trindade, 2018; Zhukov, 2018). As a result of these findings, it is possible to conclude that the composition of fatty acids produced by microalgae is influenced by metabolic pathways governed by a tolerance mechanism activated under salinity stress (Salama et al., 2013). Hence, our results suggest that *C. reinhardtii* resisted to salt stress by increasing UFA levels, especially oleic acid C18:1 and linolenic acid C18:3 and lowering the levels of SFA, notably palmitic acid C16:0 and stearic acid C18:0, which are the major constituents of FAME in microalgae under normal conditions. Figure 5 showed a positive correlation between MUFA and ROS (H_2O_2). However, SFA negatively correlated with H_2O_2.

The present study investigated the effects of salt stress on the composition of alkanes and alkenes in *C. reinhardtii*. The results are presented in Table 2 he Exposure of *C. reinhardtii* to 200 mM NaCl induced changes in the distribution of alkanes and alkenes with the detection of pentadecane and heptacene (0.29% and 0.48%) which were absent in the control. There was a decrease in alkenes, which was 1.49%

Table 2. The effect of salinity stress on the distribution of alkanes and alkenes (Area %) in *C. reinhardtii*.

	NaCl 0 mM	NaCl 200 mM
Tricosene	1.44	0.89
1-nonadecene	0.75	0.13
Heptadecene	-	0.11
Nonacosene	-	0.18
eicosene	-	0.18
Total Alkenes	2.19	1.49
Pentadecane	-	0.29
Heptadecane	-	0.48
Total Alkanes	-	0.77

Table 3. The effect of salinity stress on the distribution of sterols and other metabolites (Area %) in *C. reinhardtii*.

	NaCl 0 mM	NaCl 200 mM
Stigmasterol	0,22	-
Stigmastan-6,22-dien	1,76	-
Stigmasta-3,5-diene	-	0,76
Ergosta-4,7,22-triene	-	0,09
Cholesta-6,22,24-triene	-	1,79
Total sterols	1,98	2,64
Vit E	2,01	0,92
Trimellate	0,15	0,18
Pyridene	-	0,52
Naphtalene	-	0,05
Oxalic acid	-	0,08
Urea	-	0,21
3-Pyrazolidinone	-	0,11
Benzenamine-4-chloro-N-(2-pyridinylmethylene)	-	0,09
2-Fluorobenzoic acid Heptadecyl ester	-	0,58
2-Ethylbutyric acid octadecyl ester	-	0,57
1-Docosanol, methyl ether	-	0,53

under 200 mM NaCl compared with the control (2.19%). Alkenes such as heptadecene and very long chain alkenes such as nonacosene and eicosane were also detected under 200mM NaCl conditions (0.11%, 0.18% and 0.18%), respectively but were absent in the control. Many studies reported that *C. reinhardtii* produced C15–C17 alka(e)nes as the main hydrocarbons using cis-vaccenic acid as a precursor (Sorigué et al., 2016; Harada et al., 2021). Alkanes and alkenes are known as the main component of cuticular waxes in plants, preventing water loss and pathogen attacks etc (Wicker-Thomas and Chertemps, 2010; Sorigué et al., 2016). Furthermore, microalgae alkanes and alkenes are synthesized in the chloroplast and are accumulated in membranes showing their different roles in cell growth, cell division, photosynthesis and stress tolerance to various stress factors including salinity (Yamamori and Kageyama, 2018; Wichmann et al., 2020; Moulin et al., 2021).

A deletion mutant of alkane synthesis genes was constructed in *Synechococcus elongates PCC 7942.* The mutant was unable to produce alkanes and was salt sensitive. The growth defect under salinity stress can be restored by the overexpression of alkane synthesis genes from the halotolerant *Aphanothece halophytica* in *Synechococcus elongates* (Yamamori and Kageyama, 2018). Another study performed in vivo, confirmed that thylakoid curvature was reduced in the *Synechococcus* sp. PCC7002 mutants lacking hydrocarbons (Lea-Smith et al., 2016), concluding that hydrocarbons, especially C16–C17 alkanes and alkenes, promote membrane flexibility and facilitate curvature which required for optimal cell division (Lea-Smith et al., 2016).

Salinity changes in the culture medium affect the content of sterols and other metabolites (Ahmed et al., 2015). In the present study *C. reinhardtii* accumulated sterols (stigmasterol, ergosterol, cholesterol etc.), vitamin E, and other metabolites under 200 mM NaCl conditions (Table 3). The accumulation of sterols was slightly higher under salt stress (3.06%) compared to the control (1.98%). Sterols such as Ergosta-4,7,22-trien, Cholesta-6,22,24-triene and Stigmasta-3,5-diene were also detected under salt stress (0.09%, 1.79% and 0.76%), respectively. According to the study by Commault et al. (2019),*C. reinhardtii* contains ergosterol and 7-dehydroporiferasterol as the main membrane component. Sterols in microalgae decreases with increasing salt concentration (Francavilla et al., 2010). *D. tertiolecta* and *D. salina* accumulated higher yields of sterols (1.3% and 0.89% of dry weight), respectively at 0.6 M NaCl conditions. Sterols have a role in membrane formation and preservation, especially against hyperosmotic shocks (Francavilla et al., 2010). In microalgae, sterols play a role in cell adaptation and acclimation to changing environmental conditions (Jaramillo-Madrid et al., 2020).

4. Conclusion

The present study shows that exposure of *C. reinhadtii* to 200 mM NaCl induced physiological, biochemical and metabolomic changes. Salinity stress induced the generation of ROS with increase in H_2O_2 levels, resulting in reduced growth rate, photosynthetic pigment content (Chl a and b) and enhanced peroxidation of membrane lipids. In addition, salinity stress altered cellular metabolism, resulting in increased carbohydrate and lipid accumulation as storage molecules. Salinity stress also stimulated the antioxidative system in *C. reinhardtii*. Stressed cells accumulated carotenoids, polyphenols, carbohydrates and proline as osmoprotectant and ROS-scavenging molecules. POD and CAT activities were also higher in stressed *C. reinhardtii* to balance ROS levels in the cell. Furthermore, metabolic changes revealed a decrease in SFA, especially palmitic acid C16:0 and stearic acid C18:0, which are the major constituents of FAME in microalgae under normal conditions. Stressed cells exhibited higher UFA levels, notably oleic acid C18:1 and linolenic acid C18:3, alka€nes and sterol were also related to salt stress resistance and protection of the membrane's integrity, flexibility and facilitating curvature.

Declarations

Author contribution statement

Soufiane Fal: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Abelaziz Smouni: Conceived and designed the experiments; Wrote the paper.

Abderahim Aasfar; Reda Rabie: Analyzed and interpreted the data.

Hicham EL Arroussi: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

No data was used for the research described in the article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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