Cold stress combined with salt or abscisic acid supplementation enhances lipogenesis and carotenogenesis in Phaeodactylum tricornutum (Bacillariophyceae)

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Abstract

Microalgae have attracted interests from a range of biotechnology fields due to the variety of valuable bioactive metabolites some species can synthesise. Compounds such as ω3-fatty acids or carotenoid pigments are commercially exploited to provide the materials necessary for product formulations within the pharmacology, nutraceutical or cosmetic sectors. The co-stimulation of several compounds of interest may as such improve the cost-effectiveness of microalgal biorefinery pipelines. This study focused on the microalgal biological model species *Phaeodactylum tricornutum* (marine diatom) to investigate the effects on lipogenesis and carotenogenesis of combined stressors, here cold temperature shock (from 20 to 10°C) and addition of NaCl salt (5 mg/ml) or the phytohormone abscisic acid (4 mg/l), applied using a two-stage cultivation strategy. Results showed that cold stress with NaCl or phytohormone addition increased the neutral lipid content of the biomass (20 to 35%). These treatments also enhanced the proportions of EPA (22% greater than control) in the fatty acid profile of biomass extracts. In addition, these treatments had a stimulatory effect on carotenogenesis, especially the combination of cold stress with NaCl addition, which returned the highest production of fucoxanthin (33% increase). The gene expression of diacylglycerol acyltransferase (DGAT) and the ω-3 desaturase precursor (PTD15), as determined by real time PCR, were enhanced 4- and 16-fold relative to the control, respectively. In addition, zeaxanthin epoxidase 3 (ZEP3), involved in the xanthophyll cycle, was downregulated at low temperature when combined with abscisic acid. These results highlight the benefits of applying a combination of low temperature and salinity stress, as part of a two-stage cultivation process, to simultaneously enhance the yields of the valuable metabolites EPA and fucoxanthin in *Phaeodactylum tricornutum*.

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microalgal biological model species *Phaeodactylum tricornutum* (marine diatom) to investigate the effects on lipogenesis and carotenogenesis of combined stressors, here cold temperature shock (from 20 to 10°C) and addition of NaCl salt (5 mg/ml) or the phytohormone abscisic acid (4 mg/l), applied using a two-stage cultivation strategy. Results showed that cold stress with NaCl or phytohormone addition increased the neutral lipid content of the biomass (20 to 35%). These treatments also enhanced the proportions of EPA (22% greater than control) in the fatty acid profile of biomass extracts. In addition, these treatments had a stimulatory effect on carotenogenesis, especially the combination of cold stress with NaCl addition, which returned the highest production of fucoxanthin (33% increase). The gene expression of diacylglycerol acyltransferase (DGAT) and the ω-3 desaturase precursor (PTD15), as determined by real time PCR, were enhanced 4- and 16-fold relative to the control, respectively. In addition, zeaxanthin epoxidase 3 (ZEP3), involved in the xanthophyll cycle, was downregulated at low temperature when combined with abscisic acid. These results highlight the benefits of applying a combination of low temperature and salinity stress, as part of a two-stage cultivation process, to simultaneously enhance the yields of the valuable metabolites EPA and fucoxanthin in*Phaeodactylum tricornutum* .

Keywords: microalgae; *Phaeodactylum tricornutum* ; diatom; fatty acids; EPA, carotenoids; fucoxanthin; gene expression; abscisic acid.

Introduction

Microalgae have increasingly attracted commercial interests due to their ability to autotrophically produce metabolites with applications in the biofuel, nutraceutical, pharmaceutical, cosmetic or food and feed sectors. Among microalgae, Bacillariophyceae (diatoms) can synthesise and accumulate a variety of high-value compounds such as omega-3 fatty acids or carotenoid pigments . Diatoms use neutral lipids as their main form of intracellular carbon storage. As such, the marine species*Phaeodactylum tricornutum* has been used as model organism for studying lipid synthesis mechanisms , especially those of polyunsaturated fatty acids (PUFAs). Some diatoms can produce the long chain PUFA eicosapentaenoic acid (EPA) , a valuable ω-3 type fatty acid with anti-inflammatory, anti-viral and anti-depressive properties . Such PUFAs are considered essential nutrients for the larval development of several aquatic organisms such as fish, shrimp and bivalves that are reared by the aquaculture sector .

Xanthophyll pigments are oxygenated carotenoids with fundamental roles in plants and algae. The most important xanthophyll in diatoms is fucoxanthin, which acts as main photon receptor. There is an increasing demand worldwide for fucoxanthin as dietary supplement given its reported antioxidant, anti-obesity, anti-inflammatory, antidiabetic and antihypertensive properties (Peng et al. 2011; Wang et al. 2018).

Microalgae can modulate their lipid and pigment biosynthetic pathways under different cultivation regimes. However, such conditions can often supress cell growth, which can be limiting in the context of biorefining valuable metabolites . To overcome this drawback, two-stage cultivation approaches have been considered, whereby the stress conditions are applied later in the cultivation process once sufficient amounts of biomass have been generated. Temperature variation is a factor that can influence the synthesis of fatty acids; low temperatures in particular tend to increase the proportions of unsaturated fatty acids to maintain adequate membrane fluidity. Other factors, such as salinity, can influence the secondary metabolism of diatoms. Abscisic acid (ABA) is a stress regulator in plants that can also induce responses against environmental stress in microalgae . It has been reported to have stimulatory effects on the fatty acid content of *P. tricornutum* .

Fatty acid synthesis in *P. tricornutum* occurs in the chloroplast, where chains are assembled and then transported to the endoplasmic reticulum for further modification into PUFAs. This pathway requires the action of elongase and desaturase enzymes for double bond formation. Carotenogenesis, on the other hand, occurs via two alternative pathways based on mevalonate (MEV) and methylerythritol phosphate (MEP) (Bertrand, 2010; Kuczynska, 2015). The first assembled carotene is lycopene, which serves as substrate for the formation of other carotenes and xanthophylls (Kuczynska, 2015). Some desaturases

required for EPA biosynthesis like the Δ5 and Δ6 have been cloned and other relevant genes have also been annotated (DiatomCyc, http://www.diatomcyc.org and the Joint Genome Institute, http://genome.jgi-psf.org/Phatr2/Phatr2.home.html). Likewise, some important carotenogenesis-related genes such as the zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (DEP) have been found to be similar to those of higher plants. However, these enzymes seem to cover multiple functions in diatoms and their regulation appears fundamental for adaptation to their environment. The increasing data on *P. tricornutum* transcriptomics have paved the way for carrying out studies on the expression levels of various genes under different growing conditions (Zhang, 2020; Lopes, 2019; Conceição, 2020; Siaut, 2007; Kuczynska, 2020).

The aim of this study was to assess the effects of combining a cold temperature shock (decrease of 10°C) with the use of chemical stressors (here NaCl, ABA) applied during the stationary phase of growth of the marine diatom *P. tricornutum* CCAP1052/1. As such, the expression levels of key genes encoding for enzymes involved in lipid and carotenoid synthesis pathways were analysed 24 hours after the stressors were applied. The biomass of the cultures was also analysed in terms of lipid and pigment content 5 days after.

Materials and methods

2.1 Microalgal growth

The marine diatom *Phaeodactylum tricornutum* CCAP 1052/1 was grown in filtered-sterilised seawater enriched with Guillard f/2 with silicate . A volume of ˜4 L of culture seeded at a concentration of 0.5 mg/ml (wet weight) was incubated in a borosilicate photobioreactor at 20°C ± 1°C under a 14:10 light:dark photoperiod with a photon flux density of 80 µmol·m s^{-1}. Aeration was provided via a 0.22 µm filtered airline at a rate of 210 ml/min.

2.2 Experimental set up and harvest

The stock culture of *P. tricornutum* CCAP 1052/1 was cultivated for 20 days (stage-1). Then, 300 ml subsets of homogenised culture were placed in sterile flasks, which were incubated in triplicate under different conditions (stage-2) as detailed in Table 1. All the flasks, except the controls, were placed at 10°C under the same illumination regime used in stage-1. The low temperature treatment (LT) was tested alone and in combination with the phytohormone and salt enhancement treatments, which were selected based on other studies and preliminary trials (Garcí, 2000; Qiao, 2016; Zhang, 2020).

After 24 hours within stage-2, 10 ml of culture was collected from each flask and immediately used for RNA extraction and subsequent gene expression analysis. The *P. tricornutum* flasks (n=12) were further incubated for a total of 5 days prior to biomass harvest by centrifugation ($3000 \times g$, 10 min). The pellets were resuspended in 3 ml of ammonium formate for desalting and centrifugated again. The final pellets were freeze-dried overnight and stored at -20°C until analysis.

2.3 Pigment extraction and analysis by HPLC-UV-DAD

Pigment extraction was carried out according to McGee et al. (2018). Triplicate freeze-dried biomass samples (1.5 mg) were extracted under subdued light, grounded with glass beads in a FastPrep® FP120 for 40 sec at a speed of 4.0 m s^{-1} in a volume of 0.5 ml of ice-cold acetone (100% v/v). Then, 0.85 ml of acetone and 0.15 ml of ultrapure H_2O were added and the samples homogenised again. The extracts were then filtered (0.22 µm) and stored in amber vials at -20°C and analysed within 24 hours.

HPLC analysis was performed by reverse phase monolithic column (Merck Chromolith® Performance RP-18 100 × 4.6 mm ID) using a stepped gradient solvent program on a Shimadzu HPLC binary solvent delivery system equipped with an SPD-M20A Photo Diode Array Detector UV-Vis detector. The mobile phases and elution gradient program employed were as in McGee et al. (2018). Pigment identification was achieved by comparing retention times and UV-vis spectral fine structures to pigment standards (DHI), in-house pigment library and reference data sheets .

2.4 Neutral Lipids content using the Nile red fluorescence assay

Phaeodactylum tricornutum culture samples (n=3) were analysed using the Nile Red dye-based assay of Johnson et al. (2017) with modifications. Culture aliquots of 1 ml were centrifuged (10,000×g , 2 min) and resuspended in 500 µl of f/2 medium adjusted with 10% DMSO. Then, 200 µl of each sample was mixed with 100 µl of Nile Red (1 mg/ml in 10% DMSO:f/2) into a 96-well microplate, which was incubated at room temperature in darkness for 10 min. Fluorescence measurements were then carried out using a plate reader (Ex. 530 nm, Em. 570 nm, FLUOStar Optima spectrophotometer). A standard curve of Triolein (0.15 to 20 µM) was constructed using 20% DMSO:f/2 as diluent.

2.5 Thiobarbituric Acid Reactive Substances (TBARS) assay

Lipid peroxidation was evaluated according to Barone (2021) using triplicate freeze-dried biomass samples (2 mg), which were homogenised with 2 ml of 80:20 (v/v) ethanol:water. The suspensions were then centrifuged (538×g , 10 min) and 1 ml of supernatant was mixed with 1 ml of thiobarbituric acid (TBA) solution (20% w/v trichloroacetic acid, 0.01% butylated hydroxytoluene and 0.65% TBA). After homogenisation, the samples were heated at 80°C for 20 min, cooled and centrifuged (2690×g , 10 min). Then, 1 ml of sample was placed in a plastic cuvette and its absorbance measured at 532 nm. A calibration curve was prepared using malondialdehyde ranging from 0.025 to 0.800 mg/ml.

2.6 Lipid extraction and FAME analysis by GC-MS

Triplicate extractions were performed using 2.5 mg of freeze-dried biomass onto which 0.4 ml of methanol was added together with 0.2 ml of chloroform and 0.04 ml of ultra-pure H_2O. Nonadecanoic acid was added (3.3 mM) as an internal standard to each sample. The samples were then vortexed for 30 seconds prior to centrifugation (538×g , 10 min). The lower fraction of each tube was transferred into a new vial. The residual pellet was then processed twice more, and the resulting supernatants were combined with the first one. Anhydrous sodium sulphate was used to remove residual moisture. The final extracts were dried under a nitrogen stream prior to re-suspension in 0.25 ml of chloroform.

For the analysis, 0.1 ml of extract was incubated with 0.15 ml of TMSH® (Sigma) for 24 hours for methylation before injection in an Agilent 7890A/5975C GC-MS system fitted with a BPX70 120 m column with an internal diameter of 0.25 mm. The analytical run was based on a 5:1 split injection ratio, an inlet temperature of 250° C with a helium flow rate of 2 ml/min and the transfer line at 280°C. The oven program was set up according to Archer et al. (2021): 2 min at 50°C, ramping at 20°C/min to 16°C for 8 min, then 4°C/min up to 220°C held for 5 min, with a final step of 4°C/min to 240°C and held for 12.5 min. ChemStation software version 9.03 (Agilent, Waldbronn) was used for data process and collection.

2.7 RNA extraction and cDNA amplification

RNA was extracted from each flask 24 hours after the beginning of the stage-2 treatments. The culture samples (10 ml) were centrifuged (538×g , 10 min) and the pellets were then resuspended in 1 mL of RPE Qiagen Lysis buffer and 10% of β-mercaptoethanol. Glass beads were used for the mechanical disruption of the cells using a FastPrep® FP120 for 40 sec at a speed of 4.0 m· s^{-1} (twice). The samples were then incubated in a thermo-shaker (65°C, 300 rpm, 10 min) prior to extraction using the RNeasy Plant Mini Kit (Qiagen). The RNA concentration and quality were assessed using a Nanodrop. RNA integrity was verified by agarose gel electrophoresis (1% gel in 1X TAE buffer). The amplification for cDNA synthesis was performed according to the QuantiTect® Reverse Transcription Kit (Qiagen) following the manufacturing instruction.

2.8 Real Time-PCR analyses

Gene expression analysis was carried out by real-time qPCR for a final reaction volume of 10 µl using 100 ng of cDNA. The ribosomal protein small unit (RPS) coding gene was used as reference gene . The targeted genes

were chosen based on previous studies having shown their role in TAG, PUFA and xanthophyll biosynthesis (Zhang, 2020; Lopes, 2019; Conceição, 2020; Kuczynska, 2020) (Table 2).

Each template was amplified using the QuantiNovaSYBR(r) Green PCR kit (Qiagen) using a StepOnePlus Real Time PCR System (Applied Biosystem). The amplification program was based on Lopes et al. (2019) and contained an initial heat activation step of 95degC for 5 min followed by 40 cycles of denaturation 95degC for 15 s and combined annealing/extension (60degC for 60 s). The amplicons were subjected to a melt curve analysis using a ramp from 60 to 95degC with 0.3degC increase/step. Standard curves from serial dilutions of cDNA were performed for each primer pair to adjust the expression ratio results on the basis of the qPCR efficiency. Results were analysed using StepOne Software v2.3. The expression ratios were obtained from the Ct and efficiency values as in Pfaffl (2001) using the formula in Equation 1.

$$Gene\ expression\ ratio = \frac{(E_{\mathrm{TOI}})^{\Delta \hat{} \tau \mathrm{TOI}}}{\text{greek}(\mathrm{E_{REF}})^{\mathrm{Ct_{REF}}}} \text{(Eq. 1)}$$

Where:

E_{GOI} and E_{REF} are the calculated amplification efficiencies of the gene of interest and the reference gene, respectively.

2.9 Statistical analysis

Statistical analyses were performed using SPSS 2.6 IBM software. One-way ANOVA was used to identify significant differences among treatments with regards to pigments, fatty acids and lipid peroxidation levels. Two tails t-tests were applied to compare the real-time PCR results against the control treatment using logarithmic expression ratio values.

Results

Pigment profiling

The pigments detected in *P. tricornutum* included fucoxanthin (Fx), diadinoxanthin (Ddx), chlorophyll a (Chl a) and β-carotene (β-car) as well as diatoxanthin (Dtx) and violaxanthin (Vx) (Fig. 1).

Variations in the extract content of individual pigments across the treatments tested are documented in Figure 2. The fucoxanthin cellular content of *P . tricornutum* was significantly higher for the LT+NaCl treatment (33% greater than control) (ANOVA, F=12.3, p=0.002). The LT+NaCl and LT+ABA treatments also returned significantly greater yields of violaxanthin compared to the control (ANOVA, F=9.2, p=0.006*)*. The diadinoxanthin content was the highest for the LT+NaCl treatment (ANOVA, F=8.7, p=0.007*)* . Diatoxanthin was higher than the control in all the other treatments (p=0.05). No significant pattern was observed for chlorophyl-a and β-carotene due to substantial variation between the replicates (ANOVA, F<2.9,p >0.110).

3.2. Nile Red and TBARS assays

The three test treatments used significantly increased the lipid content of *P. tricornutum* compared to the control (ANOVA, F=50.27, p<0.001), up to 12 nmol triolein equivalents/mg DW (Fig. 3). On the other hand, no significant variation was observed in terms of lipid peroxidation levels (ANOVA, F=0.39, p=0.763).

3.3. FAME analysis by GC-MS

The main FAMEs identified in the extracts of *P. tricornutum*included C16, C16:1, C18 and C20:5 (EPA) (Table 3). The relative proportions of C14, C16, C18:1 and EPA were significantly influenced by the treatments applied during stage-2 (Table 3, Supplementary file 1). In particular, a significant increase in the proportions of EPA was observed for the LT+NaCl and LT+ABA treatments compared to the control (ANOVA, F=5.88, p=0.02).

3.4. Gene expression analysis

Real-time qPCR and gene expression analyses were performed, targeting genes involved in the biosynthetic pathways of neutral lipids and PUFAs as well as xanthophyll pigments in *P. tricornutum* (Fig. 4). DGAT expression, required for the enzyme catalysing the final reaction for TAG formation, was significantly upregulated compared to the control for the LT and LT+NaCl treatments, showing expression ratios of 4.2 and 2.6, respectively (t-test, p =0.046). The expression of PTD15 was also positively influenced by the LT, LT+NaCl and LT+ABA treatments (t-test,p =0.048). Expression levels related to pigment biosynthesis genes did not show significant results with the exception of ZEP3, which was downregulated, especially under LT+ABA (t-test, p =0.045).

Discussion

The increasing market demands for the high-value natural metabolites EPA and fucoxanthin cannot be sustainably supported by fish and seaweed harvest alone . Microalgae constitute renewable sources of such compounds, but their industrial cultivation and subsequent bioprocessing operations still require improvements to achieve greater cost-effectiveness. It has been shown that oxidative stress can trigger the production of high-value compounds in microalgae. However, stressors introduced early in microalgal cultures can impact upon cell proliferation and negatively affect the overall yields of compounds of interest . As such, two-stage growth regimes can alternatively be used so that stress factors are applied for a shorter period of time later in the growth, when more elevated cell concentrations have been reached (Sun, 2018a). Here, such approach was used to grow the marine diatom species model *P. tricornutum* under a combination of low temperature and the addition of exogenous chemicals (either NaCl or the phytohormone abscisic acid) in an attempt to stimulate PUFA and carotenoid synthesis.

4.1 Cold treatment influenced lipid biosynthesis

The 5 day-cold treatment activated in *P. tricornutum* the metabolic rearrangement typical of cold stressed cells . Results indicated that cells grown under low temperature showed a 20 to 35% increase in lipids in the biomass collected on day 25. The optimal temperature for *P. tricornutum* growth has previously been established at 20°C although this species can grow, albeit at different rates, between 5 and 30°C . In eukaryotes, triacylglycerol (TAG) synthesis takes place via the Kennedy's pathway. DGAT is the final enzyme involved in the process that catalyses the formation of TAG by acylation of diacylglycerol on the sn-3 position. This enzyme is essential for TAG production and in diatoms, including *P. tricornutum* , there are multiple copies of this gene (Chen, 2012). Upon cold exposure, cells activate a series of molecular rearrangements to cope with their new environment. For instance, lipids tend to be synthesised as a form of energy storage during slow growth or under cold conditions. This was clearly supported by our gene expression data which showed after 24 hours of low temperature treatments the upregulation of DGAT.

4.2 Combination of cold and chemical stressors stimulated PUFAs biosynthesis

A common effect of low temperature is to increase the cellular content of PUFAs as a higher degree of unsaturation is essential to maintain membrane fluidity under such condition. In this study, the LT treatment increased the proportions of EPA and C18:1 compared to the control, but not significantly so. However, this became statistically supported when the low temperature treatment was coupled with the addition of NaCl or ABA. The application of the stress led to the upregulation, within 24 hours, of PTD15, which is an ω3-desaturase involved in the PUFAs/EPA pathway and catalysing the further unsaturation of C18:2 into C18:3. In other studies having evaluated the effects of abiotic stress on the lipid metabolism of *P. tricornutum* , PTD15 was reported as one of the first genes to be upregulated after stress application. The cold-induced fatty acid remodelling response is generally fast so as to maintain cell homeostasis, as previously demonstrated in *P. tricornutum* with the lowering of temperature from 25 to 10°C causing an increase in EPA proportions within 12 to 48 hours. In the present study, the lipid content of the biomass was analysed after 5 days of treatment. The moderate increase of the unsaturation level observed under the LT treatment might be the consequence of the earlier stimulation of PUFA biosynthesis in response to cold stress.

4.3 Effect of cold and chemical stressors on carotenogenesis

The LT treatment had no effect on the pigment profile when compared to the control. However, it did when used in combination with the addition of NaCl and ABA. The LT+NaCl treatment, in particular, returned the highest Fx accumulation. The modality of Fx synthesis is not completely clear as multiple possible pathways might be involved. Previous studies showed that oxidative stress can induce Fx biosynthesis in diatoms while others showed the capability of different marine diatoms to adapt well to salinity variation (Ishika, 2017; Wang, 2018; Ishika, 2019). However, investigations specifically focusing on the effect of salinity stress on fucoxanthin production are less common, even less so when combined with other stressors. The increase in Fx along with other carotenoids in response to osmotic stress is most likely required for scavenging NaCl-induced ROS . However, our gene expression data showed that the ZEP and VDE genes were not significantly regulated.

The LT+NaCl and LT+ABA treatments both stimulated Vx and Dtx biosynthesis relative to the control. The de-epoxidation of xanthophylls is a mechanism known in diatoms for considerably reducing the membrane-damaging effects of ROS on thylakoid membranes and preventing lipid peroxidation (Lepetit, 2010; Lepetit, 2012). The LT+ABA treatment showed a significant 3.3-fold downregulation of the ZEP3 gene. The gene expression results of these experiments relative to the xanthophyll cycle are unclear. Two types of xanthophyll cycle have been described in diatoms: the violaxanthin cycle (VAZ cycle) and the diadinoxanthin cycle (DD cycle) (Kuczynska, 2015). These are based on the same mechanism of de/epoxidation through which the interconversion of different xanthophylls takes place. ZEP and VDE genes are classically reported to be specific for the VAZ cycle in plants but in diatoms, they might act on multiple substrates and thus also be involved in the diadinoxanthin cycle (Kuczynska, 2020; Blommaert, 2021). The downregulation of ZEP 3 might justify the accumulation of Vx as well as Dtx, as observed in this study, or possibly both of them. It still remains unknown which isoforms of both enzymes previously identified in *P. tricornutum* are engaged in the DD or VAZ cycles (Kuczynska, 2015).

4.4 General antioxidant response to stressors

The increase in the relative proportions of PUFAs observed in the*P. tricornutum* extracts following the LT+NaCl and LT+ABA treatments may result from oxidative stress reaction mechanisms. Reactive oxygen species (ROS) can cause lipid peroxidation, inhibit photosynthesis, damage DNA, proteins, lipids, and can lead to cell death. The accumulation of compounds such as PUFAs in plants and algae can help overcoming the effects of ROS (Sun, 2018b).

Osmotic stress can induce oxidative stress in plants and algae . Under high salinity, ROS production increases as a result of alterations to electron transport and the accumulation of photo-reducing power . Previous experiments showed that osmotic duress can stimulate lipid accumulation in marine diatoms. In *Dunaliella salina* , a halotolerant chlorophyte, 3.5 M NaCl supplementation also induced the accumulation of PUFAs. These studies showed that NaCl can influence the expression of enzymes involved in the biosynthesis of PUFAs probably for cells to better withstand pressure on the membranes (Chen, 2009). Our data indicated that the LT+NaCl treatment increased the proportions of EPA and also stimulated the expression of the PTD15 gene (8.6-fold). However, peroxidation levels in the cultures exposed to LT+NaCl did not show any significant difference compared to the other treatments.

ABA is a stress related hormone that is well conserved among plants and algae and that acts as a signalling molecule. In plants, it can activate several transcription factors essential for stress response. This study provided further evidence of ABA stimulating lipid accumulation, as in other microalgae and higher plants (Lu, 2015; Han, 2018; Zhang, 2020; Yang, 2020; Sivaramakrishnan, 2020; Tuteja, 2007; Sun, 2018a). This may be related to ABA triggering the production of ROS, which can influence several biosynthetic pathways, including lipid production (Cho, 2009; Postiglione, 2020; Sun, 2018b). However, ABA-induced ROS are generally found at a low concentration just sufficient to elicit responses; TBARS indeed did not show any relevant increment of peroxidation levels compared to the control.

The similarity of the effects of LT+NaCl and LT+ABA could be explained by the fact that ABA is an important messenger under salinity stress in plants . ABA biosynthesis, subsequently to cells experiencing

osmotic stress, has been described in several microalgal phyla such as *Chlorophyta, Chromophyta, Cyanophyta* or *Cryptophyta* . An overlap in the expression pattern of stress-related genes after cold, drought, salinity or ABA application have been observed, suggesting that various stress signals and ABA share common elements in the signalling pathway responses . As such, recent findings have highlighted the interplay between salinity, oxidative stress, and lipid accumulation in microalgae (Qiao, 2021; Barone, 2021).

Conclusion

The combination of cold treatment and chemical stressors led to the accumulation of high-value metabolites in *P. tricornutum* . The LT treatment activated the transcription of genes involved in TAG and PUFA biosynthesis but the accumulation of PUFAs became significant only when LT was combined with NaCl or ABA. These treatments also stimulated carotenogenesis, leading to an increase in the two valuable microalgal metabolites EPA and fucoxanthin. This mixed stressor strategy applied as part of a 2-stage cultivation regime shows promises for the further enhancement of high-value compounds in marine diatoms.

Table 1. Treatments applied during the second stage cultivation (5 days) of the marine diatom *Phaeodactylum tricornutum* (CCAP 1052/1).

Table 2. List of selected genes involved in TAG, PUFA and xanthophyll biosynthesis in *Phaeodactylum tricornutum* and primer sequences used for gene expression analysis by quantitative real-time PCR.

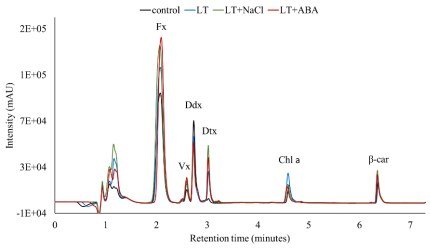
Table 3. GC-MS based variations in the relative proportions of the main FAMEs in extracts of *Phaeodactylum tricornutum* (CCAP 1052/1) for each stage-2 cultivation treatment tested. Letters indicate homogenous subsets for each fatty acid (p [?]0.05).

Figure 1. Overlaid HPLC-UV chromatograms of *Phaeodactylum tricornutum* (CCAP 1052/1) extracts for each stage-2 cultivation treatment tested.

Figure 2. Variation in the fucoxanthin (Fx), violaxanthin (Vx), diadinoxanthin (Ddx), diatoxanthin (Dtx), chlorophyll a (Chl a) and β-carotene (β-car) content of *P. tricornutum* (CCAP 1052/1) extracts (normalised by maximum peak area for each pigment) for each stage-2 cultivation treatment tested. Letters indicate homogenous subsets for each pigment (p[?]0.05).

Figure 3. Neutral lipid content (A, Red Nile assay) and lipid peroxidation levels (B, TBARS assay) in samples of *Phaeodactylum tricornutum* (CCAP 1052/1) for each stage-2 cultivation treatment tested. Letters indicate homogenous subsets (p[?]0.05).

Figure 4. Relative expression ratios of genes involved in fatty acid (A) and pigment (B) biosynthetic pathways in *Phaeodactylum tricornutum* (CCAP 1052/1) for each stage-2 cultivation treatment tested compared to the control. Stars indicate significant differences relative to the control (p <0.05).



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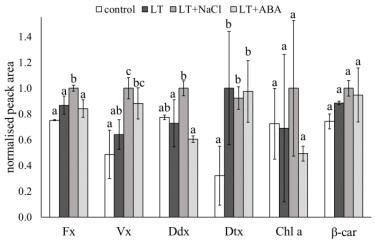

Figure 2. Variation in the fucoxanthin (Fx), violaxanthin (Vx), diadinoxanthin (Ddx), diatoxanthin (Dtx), chlorophyll a (Chl a) and β-carotene (β-car) content of *P. tricornutum* (CCAP 1052/1) extracts (normalised by maximum peak area for each pigment) for each stage-2 cultivation treatment tested. Letters indicate homogenous subsets for each pigment ($p \leq 0.05$).

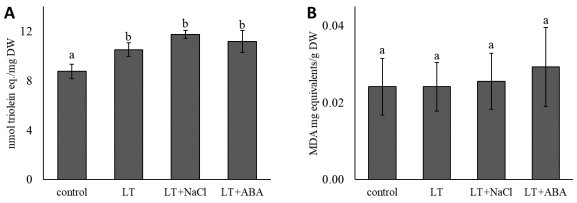

Figure 3. Neutral lipid content (A, Red Nile assay) and lipid peroxidation levels (B, TBARS assay) in samples of *Phaeodactylum tricornutum* (CCAP 1052/1) for each stage-2 cultivation treatment tested. Letters indicate homogenous subsets (p≤0.05).

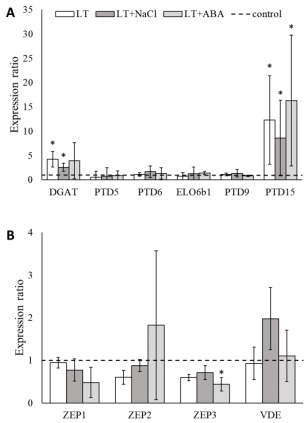

Figure 4. Relative expression ratios of genes involved in fatty acid (A) and pigment (B) biosynthetic pathways in *Phaeodactylum tricornutum* (CCAP 1052/1) for each stage-2 cultivation treatment tested compared to the control. Stars indicate significant differences relative to the control (p<0.05).

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`PtGE tables.docx` available at https://authorea.com/users/474109/articles/564059-cold-stress-combined-with-salt-or-abscisic-acid-supplementation-enhances-lipogenesis-and-carotenogenesis-in-phaeodactylum-tricornutum-bacillariophyceae