Phytoene and phytofluene overproduction by *Dunaliella salina* using the mitosis inhibitor chlorpropham

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A B S T R A C T

The halotolerant chlorophyte microalga, *Dunaliella salina*, is one of the richest sources of carotenoids and will accumulate up to 10% of the dry biomass as β-carotene, depending on the integrated amount of light to which the alga is exposed during a division cycle. Red light also stimulates β-carotene production, as well as increases the *9-cis* β-carotene/*all-trans* β-carotene ratio. In this paper we investigated the effects of chlorpropham (Iso-propyl-N (3-chlorophenyl) carbamate, CIPC), with and without red light, on carotenoid accumulation. Chlorpropham is a well-known carbamate herbicide and plant growth regulator that inhibits mitosis and cell division. Chlorpropham arrested cell division and induced the massive accumulation of colourless phytoene and phytofluene carotenoids, and, to a much lesser extent, the coloured carotenoids. The chlorophyll content also increased. When phytoene per cell accumulated to approximately the same level with chlorpropham as with norflurazon, a phytoene desaturase inhibitor, coloured carotenoids and chlorophyll increased with chlorpropham but decreased with norflurazon. Cultivation with chlorpropham under red LED light for 2 days did not affect the content of β-carotene, but phytoene was 2.4-fold the amount that was obtained in cultures under white LED light and the *9-cis* β-carotene/*all-trans* β-carotene ratio increased from 1.3 to 1.8. With norflurazon, red LED light boosted the contents of both phytoene and β-carotene, 2-fold and 1.3-fold respectively compared to those under white LED light, and the ratio of *9-cis* β-carotene/*all-trans* β-carotene reached 3.8. The results are discussed in terms of disruption by chlorpropham of synchronised control between nuclear and chloroplast events associated with carotenoid biosynthesis. Since phytoene and phytofluene are colourless carotenoids which are sought after for the development of nutricosmetics and other health/beauty products, the results also present as a new method with low toxicity for production of colourless carotenoids based on the cultivation of *D. salina*.

1. Introduction

Carotenoids are conjugated isoprenoids with a long system of conjugated double bonds and are synthesised by all photosynthetic organisms for light-harvesting and for photo-protection. They are also precursors for the biosynthesis of phytohormones which control apocarotenoid signalling metabolites that mediate chloroplast to nucleus communications (for reviews see [1–6]).

Phytoene and phytofluene are among the few carotenoids that are colourless and may provide antioxidant activity, anticarcinogenic activity, anti-inflammatory activity, and protection against UV-induced damage, with phytoene absorbing maximally in the UVB region and phytofluene in the UVA region [7,8]. They can be ingested or topically applied and are of great interest in the nutricosmetic field for their skin health and aesthetic benefits. Phytoene and phytofluene are derived from the ubiquitous isoprenoid precursor, geranylgeranyl diphosphate (GGPP); phytoene synthase (PSY) is the enzyme responsible for conversion of GGPP to phytoene, the first carotenoid product, and phytoene desaturase (PDS) is responsible for catalysing formation of phytofluene from phytoene (See Fig. 1).

The halotolerant chlorophyte microalga, *Dunaliella salina*, is one of the richest sources of carotenoids and accumulates up to 10% of the dry biomass as β-carotene [11–16]. This is packed in so-called 'βC-plastoglobuli' which form in the inter-thylakoid spaces of the chloroplast when the alga is exposed to high light, or to deprivation of nutrients or other growth limiting conditions such as iron depletion, very high salt >1.5 M, or sub-optimal temperature [11,13,15,17–19], and depends on the integral irradiance per cell division cycle [17,20]. Red light has also been shown to promote carotenoid accumulation [21,22].

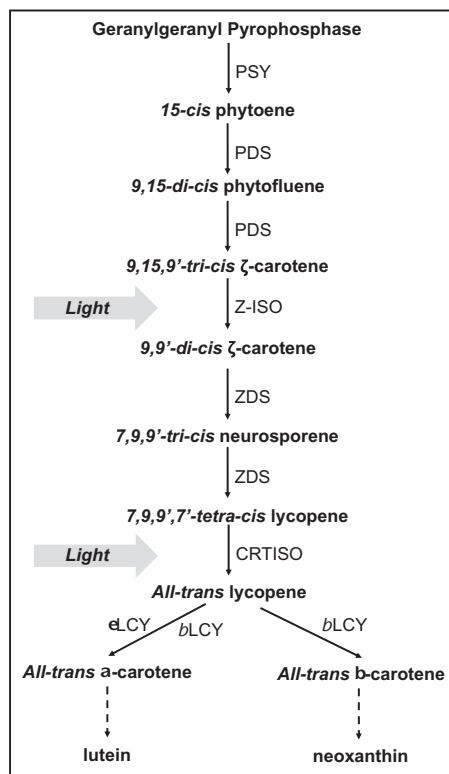The pathway for β-carotene biosynthesis and accumulation in

Fig. 1. A generally acknowledged biosynthetic pathway of carotenoids in plants and algae [5,9,10]. PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; εLCY, lycopene ε-cyclase; βLCY, lycopene β-cyclase.

D. salina takes place in the chloroplast [18,23], which divides once per nuclear cell cycle. *D. salina* is thought to use the same plastidic methylerythritol-4-phosphate (MEP) pathway as in higher plants [4,18,19,23–25]. In brief, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are synthesised as building blocks to form geranylgeranyl diphosphate (GGPP). GGPP is a precursor of both chlorophyll *a*nd phytoene but is channelled to make phytoene as a result of the interaction between GGPP synthase 11, a soluble protein found in the stroma, and the thylakoid membrane-associated enzyme, PSY. PSY catalyses the head-to-head condensation of two GGPP molecules to yield 15-*cis* phytoene in the first committed reaction of carotenogenesis. Thereafter, a series of desaturation, cyclization and hydroxylation reactions take place, all of which are complex redox reactions dependent on competent membrane structures [4,26] and access to appropriate cofactors. In higher plants the main rate-determining reaction controlling carbon flux through the carotenoid biosynthetic pathway is catalysed by PSY, which is upregulated during photomorphogenesis via a phytochrome-mediated red-light pathway [25–28]. The same seems likely in *D. salina* [21]. Desaturation reactions catalysed by PDS and ζ-carotene desaturase (ZDS) in higher plants are also important in controlling flux through the pathway and these require coupled electron transfer with a plastidial terminal oxidase (PTOX) and plastoquinone, as has been proposed for *D. salina* [21]. Significantly, PDS is also associated with plastid to nucleus (retrograde) signalling to co-ordinate the expression of genes in the nuclear and plastid genomes that are involved in the synthesis of chloroplast proteins [5,29].

Chlorpropham (Iso-propyl-N(3-chlorophenyl) carbamate, CIPC) is a well-known carbamate herbicide and plant growth regulator that inhibits mitosis and cell division by interfering with the organisation of the spindle microtubules; multiple spindles are formed and multiple nuclei result [30]. In this paper we explore the use of chlorpropham to inhibit *D. salina* nuclear cell division and the effects that its use has on the

carotenoid accumulation in the chloroplast. In parallel, we compare the effects of this inhibitor with those caused by norflurazon, a known PDS inhibitor, which blocks de novo synthesis of phytofluene and hence β-carotene and reduces chloroplastic oxygen dissipation via a plasto-quinol:oxygen oxidoreductase (PTOX), but does not to block the first committed step of the carotenoid pathway, namely phytoene synthesis [21,31,32].

2. Materials and methods

2.1. Algal strain and cultivation

Dunaliella salina strain CCAP 19/41 was obtained from the Marine Biological Association, UK (MBA). Chlorpropham and norflurazon were obtained from Sigma-Aldrich, Inc. (Merck KGaA, Darmstadt, Germany) and prepared as 1 M stock solutions in ethanol before use. Unless otherwise stated, algae were cultured in 500 ml Modified Johnsons Medium [33] containing 1.5 M NaCl and 1 mM N (KNO$_3$), in Erlenmeyer flasks in an ALGEM Environmental Modelling Labscale Photobioreactor (Algenuity, UK) at 25 °C as previously described [21]. Cultures were illuminated with either continuous white LED light or continuous red LED light supplied in the photobioreactor (light spectrum shown in Supplemental data Fig. S1).

For treatments with herbicides, *D. salina* cultures were grown to log phase under white LED light at ~200 μmol m^{-2} s^{-1} then cultures were divided into triplicate sets of flasks and either chlorpropham or norflurazon was added to 2 sets and the third served as control. Cultures were maintained for 6 days further at 25 °C under either white or red (625–680 nm) LED light at ~200 μmol m^{-2} s^{-1}. Different concentrations of chlorpropham (0.1–100 μM) and different concentrations of norflurazon (1–10 μM) were tested to determine the optimal working concentration for each herbicide. Different light intensities (50–1500 μmol m^{-2} s^{-1}) were tested to study the effect of light intensity. The cell density of the cultures was determined by counting the cell number of cultures using a haemocytometer, after fixing cells with 2% formalin.

2.2. Carotenoids analysis

Algal biomass was collected from 15 ml cultures by centrifugation at 3000 ×*g* for 10 min and pigments were extracted in the dark from the wet pellets (~90% water) with either 10 ml absolute ethanol or with 10 ml methyl tert-butyl ether (MTBE): methanol (20/80 *v/v*) after 20 s of sonication as described in our previous study [20]. The same yields of all non-polar carotenes, and more polar xanthophylls and chlorophyll pigments were obtained using either solvent, and colourless (white) biomass residues remained. Each sample of biomass collected was analysed in triplicate. Spectrophotometry was used to assess the amounts of total chlorophylls and total coloured carotenoids in the cultures as previously described [21] using a Jenway 6715 UV/Vis spectrophotometer (Cole-Parmer, Staffordshire, UK). The concentration of individual carotenoids was determined using standards of *all-trans* β-carotene, *all-trans* α-carotene, lutein, zeaxanthin and phytoene isomers purchased from Sigma-Aldrich (UK), after separation by High-Performance Liquid Chromatography using a YMC30 250 × 4.9 mm I. D S-5 μ HPLC column (YMC, Europe GmbH) with Diode-Array Detection (HPLC-DAD) (Agilent Technologies 1200 series, Agilent, Santa Clara, United States) at wavelengths of 280 nm (phytoene), 355 nm (phytofluene), 450 nm (β-carotene, α-carotene, lutein and zeaxanthin), and 663 nm (chlorophylls). The identification of peaks corresponding to phytoene and phytofluene after HPLC-DAD was confirmed after structural determination by Ultra-Performance Convergence Chromatography coupled with Mass Spectrometry detection (UPC^2 - MS) and on the basis of proton and carbon chemical shift values obtained using Nuclear Magnetic Resonance (NMR), as detailed in [34]. HPLC was at 25 °C with an isocratic solvent system of 80% methanol: 20% methyl tert-butyl ether and flow rate of 1 ml min^{-1} at a pressure of 78 bar as previously
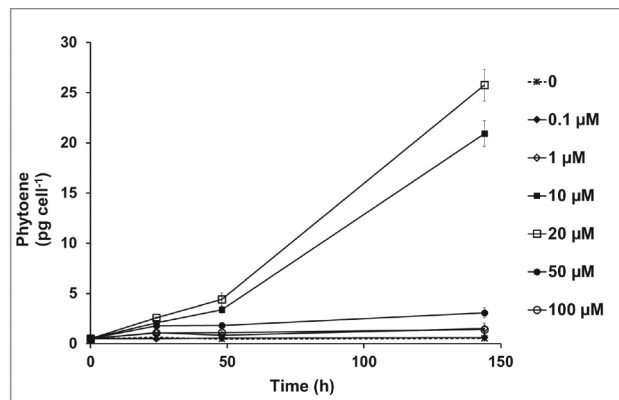
described [22].

2.3. Data analysis

Each experiment was carried out at least in triplicate ($n \geq 3$). The collected data were analysed in R by one-way analysis of variance (ANOVA) with posterior Dunnett's test compared to control cultures
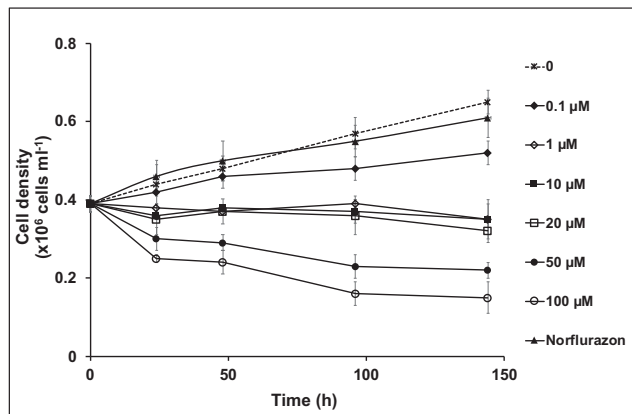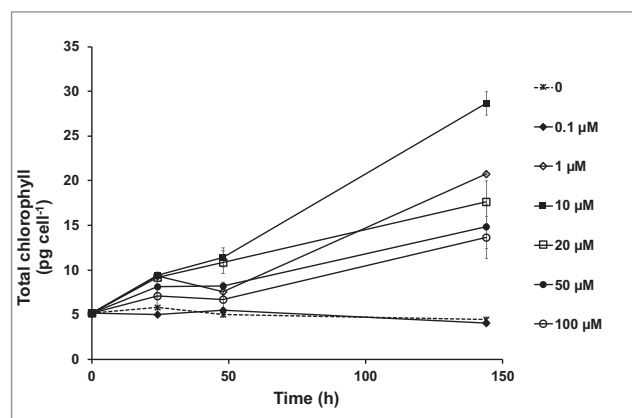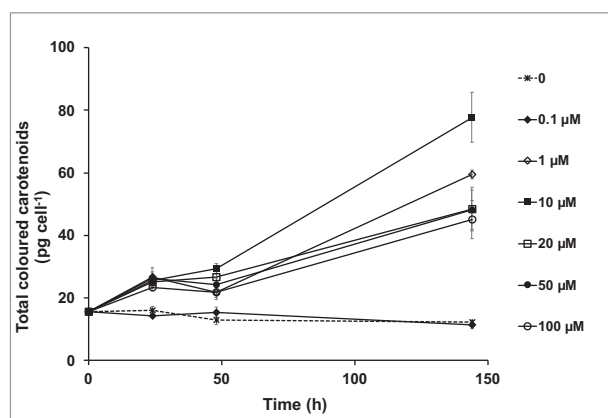

(A)

(B)

(C)

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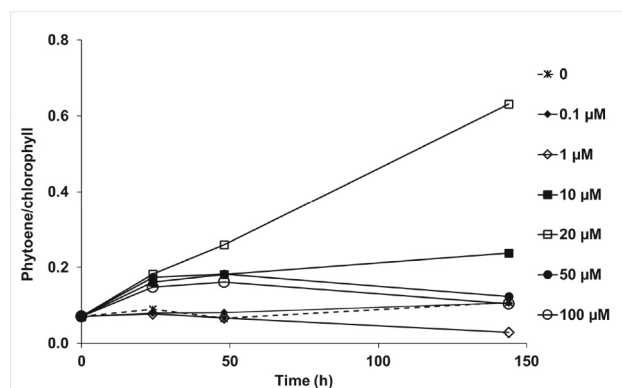
(E)

Fig. 2. Kinetic performance of *D. salina* cultures treated with different concentrations of chlorpropham (0, 0.1, 1, 10, 20, 50 and 100 μM) or 5 μM norflurazon. Cultures were maintained under white LED light at ~200 μmol m^{-2} s^{-1} for 6 days. Each treatment condition was repeated in triplicate ($n = 3$). (A) Cell density, (B) cellular content of phytoene, (C) cellular content of chlorophyll, (D) total coloured carotenoids, (E) ratio of the amounts of phytoene/chlorophyll. Total coloured carotenoids measured by spectrophotometry. Error bars show ±SD, $N = 3$.

3. Results

3.1. Effects of chlorpropham concentration on cell division and on the accumulation of phytoene, chlorophyll and coloured carotenoids

The addition of chlorpropham to cultures of *D. salina* within the applied concentration range 1–20 μM inhibited cell division during the subsequent 6 days of culture (see Fig. 2A). At higher chlorpropham concentrations (50 μM or more) cells ceased to divide and lysed with time, whilst at 0.1 μM chlorpropham, cell division was only partially inhibited. The effects on cell division were the same under red or white LED light (data not shown). Norflurazon, at the optimum concentration for phytoene accumulation caused by PDS inhibition (5 μM determined by prior experiment), by contrast, had no effect on cell division, as expected. Unexpectedly, inhibition of cell division by chlorpropham was accompanied by the massive accumulation of phytoene, a lesser accumulation of phytofluene and, to a lesser extent, the coloured carotenoids. The amount of phytoene that accumulated depended on the concentration of chlorpropham applied to the cultures. Fig. 2B shows the phytoene content expressed per cell and Fig. 2E, the ratio of phytoene and chlorophyll content, for cultures cultivated under white LED light at 200 μmol m^{-2} s^{-1} at different working concentrations of chlorpropham (0.1, 1, 10, 20, 50 and 100 μM) (see also Supplemental Fig. S2). After initial acclimation of cultures to culture conditions during the first 24 h, phytoene accumulated with increasing concentration of chlorpropham up to a maximum value of 20 μM but decreased with time at the high (50 μM or more) concentrations which caused cells to lyse (Fig. 2A). Among the concentrations tested, 20 μM chlorpropham gave the highest cellular content (Fig. 2B) and yield of phytoene and phytofluene over the cultivation period under white LED light. After 6 days, the final phytoene concentration in cultures treated with 20 μM chlorpropham was 3.60 ± 0.15 mg L^{-1} (~1.8% phytoene, DW basis; 2.25% phytoene, AFDW basis, with ~20% ash content in the biomass) compared to 0.36 ± 0.07 mg L^{-1} (0.1–0.2% phytoene, DW basis; 0.125–0.25% phytoene, AFDW basis) in control cultures (10-fold the original value). The appearance of the cells treated with 20 μM chlorpropham compared to control cells is shown in Supplemental Fig. S3.

Chlorophyll content and total coloured carotenoids i.e. β-carotene, α-carotene, lutein and zeaxanthin, measured by spectrophotometry, also increased with increasing applied concentration of chlorpropham within the range 1–10 μM (Fig. 2C, Fig. 2D). With increasing concentration beyond 10 μM, the cellular content of each of these pigments decreased. At 0.1 μM chlorpropham, none of the carotenoids or chlorophyll accumulated compared to untreated cells (Fig. 2B, Fig. 2C, Fig. 2D), even though cell division was partially inhibited (Fig. 2A).

Table 1 compares pigment composition in *D. salina* cultures treated with 20 μM chlorpropham or 5 μM norflurazon. Both chlorpropham and norflurazon caused an increase in the cellular concentration of total carotenoids compared to untreated cells (Table 1). In norflurazon-treated cells, the net rate of carotenoid biosynthesis over the 6-day time course increased to ~1.3 fold the value in untreated cells and the major carotenoid was phytoene, which accumulated massively (~44-fold the value obtained in untreated cells). After 6 days growth, the ratio of total carotenoid: chlorophyll reached 6.8 in norflurazon-treated cells compared to 4.6 in untreated.

Treatment with chlorpropham also resulted in a massive accumulation of total carotenoids (1.8-fold by day 6, see Table 1) compared to untreated cells. However, unlike the situation with norflurazon, upon treatment with chlorpropham, chlorophyll also massively accumulated (2.4-fold the value in untreated cells) such that the ratio of total carotenoid: chlorophyll decreased compared to untreated cells, to 3.5. In chlorpropham-treated cells, the major accumulated carotenoid was phytoene, (see Supplemental Fig. S2), which increased to~47-fold the value in untreated cells. Phytofluene also accumulated (~7-fold the value obtained in untreated cells). Lutein and zeaxanthin together increased in proportion to the increase in total carotenoid content. By

Table 1

Pigment composition of major carotenoids and chlorophylls in *D. salina* cultures treated with 20 μM chlorpropham or 5 μM norflurazon for 6 days. Cultures were maintained under continuous white LED light at ~200 μmol m^{-2} s^{-1}. Under these conditions, 5 μM norflurazon was determined by prior experiment as the optimal concentration required for maximal accumulation of phytoene. Each culture condition was set up at least in triplicate ($n \geq 3$). Data presented are mean ± standard deviations. *Values estimated for total carotenoids in the table are the sum of phytoene, phytofluene, β-carotene, α-carotene, lutein and zeaxanthin. **Values estimated for total coloured carotenoids are the sum of β-carotene, α-carotene, lutein and zeaxanthin.

Cellular content (pg cell^{-1})	Starting culture (T0)	Control (No herbicide)	Chlorpropham 20 μM	Norflurazon 5 μM
Phytoene	0.60 ± 0.12	0.55 ± 0.01	25.76 ± 1.58	24.38 ± 3.38
Phytofluene	0.09 ± 0.01	0.18 ± 0.00	1.27 ± 0.08	0.02 ± 0.00
β-carotene	20.76 ± 0.33	31.39 ± 0.72	31.41 ± 0.85	18.82 ± 0.12
α-carotene	0.66 ± 0.12	0.61 ± 0.04	0.68 ± 0.04	0.55 ± 0.03
Lutein	0.65 ± 0.07	0.63 ± 0.01	1.10 ± 0.02	0.48 ± 0.04
Zeaxanthin	0.48 ± 0.10	0.37 ± 0.01	1.13 ± 0.06	0.50 ± 0.01
*Total carotenoids	23.24 ± 0.39	33.73 ± 0.72	61.35 ± 1.80	44.75 ± 3.38
**Total coloured carotenoids	22.55 ± 0.37	33.00 ± 0.72	34.32 ± 0.85	20.35 ± 0.13
Total chlorophylls	8.24 ± 0.54	7.38 ± 1.08	17.60 ± 2.41	6.56 ± 0.85
Ratio total carotenoids: chlorophylls	2.8	4.6	3.5	6.8
Ratio β-carotene: chlorophyll	2.5	4.3	1.8	2.9
Ratio phytoene: coloured carotenoids	0.03	0.02	0.82	1.30

contrast, β-carotene contents were the same in both treated and untreated cells and were seemingly unaffected by chlorpropham treatment. β-carotene represented ~93% of the total carotenoid content in untreated cells compared to just over 50% in treated cells.

3.2. Effects of light intensity on phytoene accumulation with chlorpropham treatment

Phytoene production with chlorpropham also depended on the intensity of the applied light. Fig. 3 shows phytoene production under different light intensities (50, 100, 200, 500, 1000 and 1500 μmol m^{-2} s^{-1}) of white LED light with 20 μM chlorpropham. The higher the light intensity, the higher the cellular phytoene content and total yield (Fig. 3A). After 4 days cultivation, the phytoene content reached above 30 pg cell^{-1} under 1500 μmol m^{-2} s^{-1} compared to less than 1 pg cell^{-1} at time zero, and the phytoene yield in the cultures reached above 8 mg L^{-1}. However, the kinetic profiles for chlorophyll content and total coloured carotenoids differed to that of phytoene and after the first 20 h, during which time both chlorophyll and total coloured carotenoids declined, chlorophyll content increased but the rate of increase declined with increasing light intensity (Fig. 3B). The rate of increase in total coloured carotenoids after the first 20 h was negligible for all light intensities (Fig. 3C). After 4 days with 50 μmol m^{-2} s^{-1} white LED light, the sum of colourless and coloured carotenoids was calculated to be ~32 pg cell^{-1}, but with 1500 μmol m^{-2} s^{-1}, increased by 88% to ~60 pg cell^{-1}.
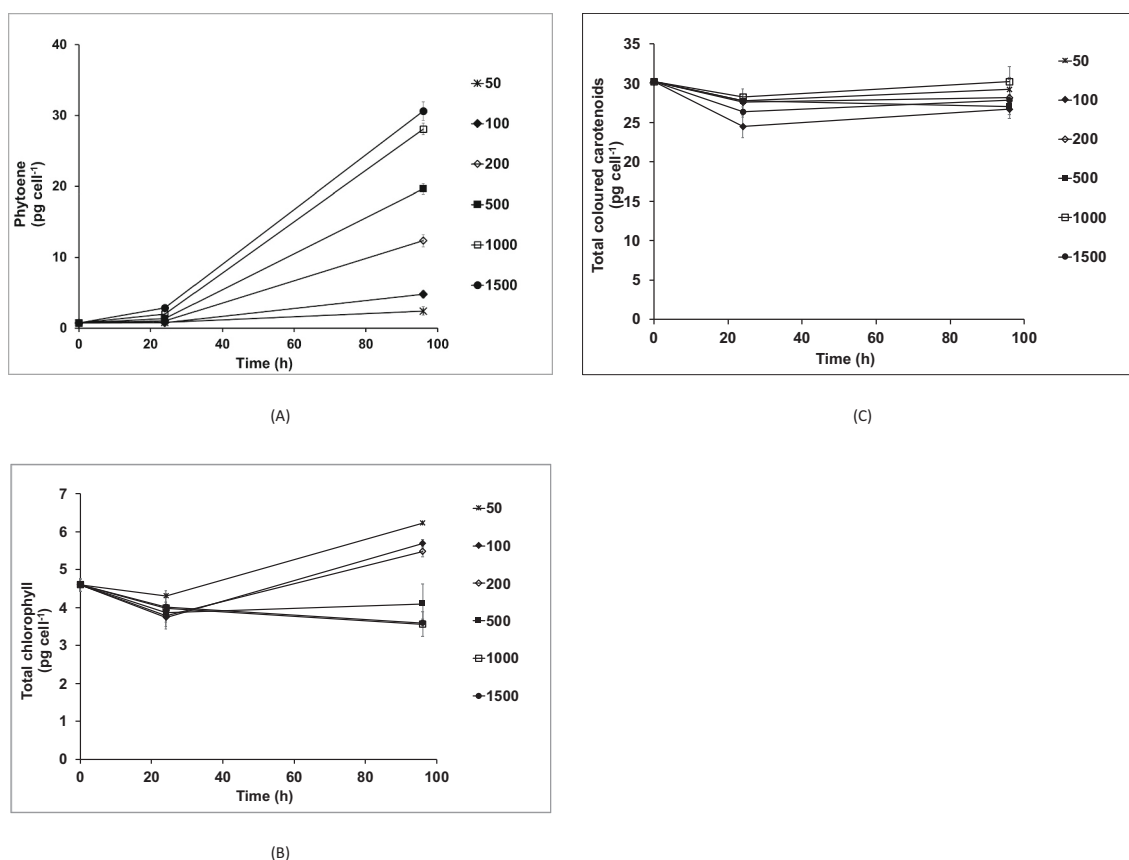
Fig. 3. (A) Cellular content of phytoene, (B) cellular content of chlorophyll and (C) cellular content of total coloured carotenoids in *D. salina* cultures treated with 20 μM chlorpropham under different intensities of continuous white LED light (50, 100, 200, 500, 1000 and 1500 μmol m^{-2} s^{-1}). Each treatment condition was repeated in triplicate ($n = 3$). Error bars show ±SD, $N = 3$.

3.3. Effects of red light on phytoene accumulation with chlorpropham treatment

Cultivation under red light is known to enhance carotenoid accumulation [21,22]. Fig. 4A shows that cultures with 0.1 mM N in the culture medium had a significantly higher phytoene content under red LED light compared to cultures under white LED light, but the effect decreased with increasing N content. With 1 mM N after 6 days cultivation, the phytoene content was only slightly higher under red LED light compared to white ($0.01 < p \leq 0.05$ between white and red light and compared to 0.1 mM N). When chlorpropham was added to cultures with 1 mM N, the content of phytoene under red LED light doubled compared to cultures maintained under white LED light, with the same concentration of chlorpropham (Fig. 4B). The optimal concentration of chlorpropham required for maximal phytoene accumulation under red LED light at ~200 μmol m^{-2} s^{-1} was 10 μM, whereas in white LED light it was 20 μM. Under white LED light, the cellular content of phytoene in cultures treated with 20 μM chlorpropham reached 25.76 ± 1.58 pg cell^{-1}, while under red LED light, it reached 51.88 ± 4.53 pg cell^{-1}.

The cellular pigment composition of cultures treated by either 10 μM chlorpropham or norflurazon under red LED light compared to white LED light for 48 h is shown in Table 2. Under white light, with no herbicide, the *9-cis* β-carotene/*all-trans* β-carotene ratio increased from 1.3 to 1.7 with time but under red light, the ratio reached 2.4. When cell division was arrested with chlorpropham, the *9-cis* β-carotene/*all-trans* β-carotene ratio under white light remained at 1.3. Under red light this increased to 1.8. However, the content of total carotenoids per cell increased with chlorpropham under white light by 60% and under red light by 95% compared to untreated cells. With norflurazon under white light, the content of total carotenoids remained the same as in untreated

cells, β-carotene per cell decreased by 31% compared to untreated cells and phytoene increased, 8.95-fold. Under red LED light, phytoene increased yet further, and was 18.25-fold the value of that in untreated cells. Surprisingly, the content of total β-carotene with norflurazon also increased under red LED light, and was >25% greater compared to under white LED light. The total content of carotenoids under red light increased by 43% compared to untreated cells and the ratio of *9-cis* β-carotene/*all-trans* β-carotene increased to 3.8.

4. Discussion

Chlorpropham is a well-known carbamate herbicide and plant growth regulator that inhibits mitosis and cell division by interfering with the organisation of the spindle microtubules. In the present work we found that chlorpropham arrested nuclear cell division in *D. salina* cells and, within the concentration range 1–20 μM, did not cause evident cell lysis. Cultivation with chlorpropham was therefore expected to increase the concentration of β-carotene in cells because the integral quantity of light to which the algae were exposed during cultivation would be increased [11,17,32]. Cultivation with chlorpropham was also expected to increase the *9-cis/all-trans* ratio, for the same reason [35].

Unexpectedly, the colourless precursor, phytoene, not β-carotene, accumulated massively when cell division was arrested with chlorpropham (47-fold with 20 μM chlorpropham after 6 days under white LED light, compared to untreated cells, see Table 1 and Fig. 2B). Phytofluene also increased, but to a lesser extent (7-fold) and, with HPLC methods (Table 1) phytoene and phytofluene together now represented 44% of total carotenoids after 6 days cultivation with 20 μM chlorpropham, compared to ~2% in untreated cells. The coloured carotenoids increased based on estimates using absorbance at 480 nm (Fig. 2D),
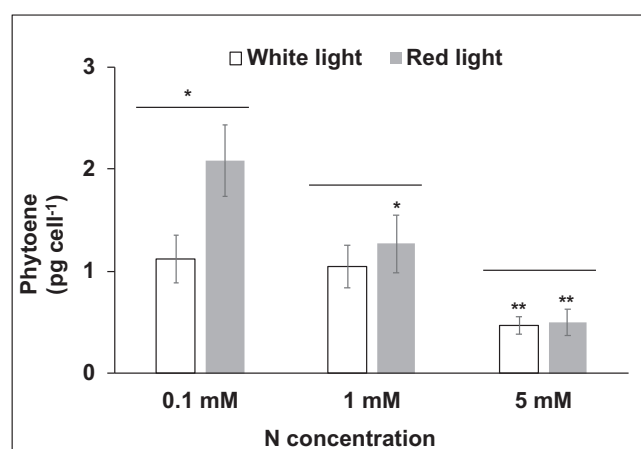
(A)
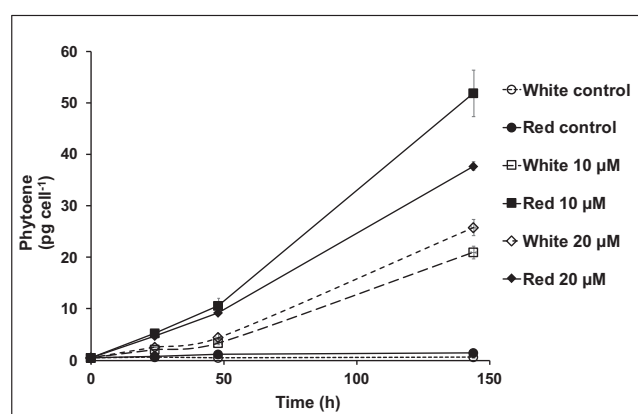
(B)

Fig. 4. (A) Cellular content of phytoene in *D. salina* cultures grown with various concentrations of N (0.1, 1 and 5 mM KNO$_3$) in Modified Johnsons medium without herbicide treatment. Cultures were maintained under red or white LED light at 200 µmol m^{-2} s^{-1} for 6 days. Each treatment condition was repeated at least in triplicate ($n \geq 3$). Results were analysed by one way ANOVA compared to 0.1 mM N and between white and red light. Asterisks represent different levels of significance (***$0 < p \leq 0.001$, **$0.001 < p \leq 0.01$, *$0.01 < p \leq 0.05$). (B) Cellular content of phytoene in *D. salina* cultures grown in 1 mM KNO$_3$ in Modified Johnsons medium and treated with either 10 or 20 µM chlorpropham under red or white light at 200 µmol m^{-2} s^{-1} for 6 days. Each treatment condition was repeated in triplicate ($n = 3$). Error bars show ±SD, $N = 3$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

but the major coloured carotenoid, β-carotene was the same after 6 days as in untreated cells (compare Table 1 with Fig. 2D). As has been shown for norflurazon [32], the yields of colourless and coloured carotenoids depended on chlorpropham concentration (Fig. 2). Since total carotenoids increased when cell division was arrested with chlorpropham (Table 1 and Table 2), and moreover, increased with increasing light intensity (Fig. 3), these data confirmed that the amount of phytoene [35] or β-carotene [11,17,23,32,35] that accumulates in *D. salina* is positively correlated with the integral irradiance to which the cells are exposed during a division cycle. However, Ben-Amotz et al [35] also showed that the *9-cis/all-trans* β-carotene ratio correlated positively with the integral irradiance received by the algal culture during a division cycle as well, whereas in the present work, under white light, the *9-cis/all-trans* β-carotene ratio did not increase with chlorpropham; it remained at the same value as at the outset of the experiment (1.3, Table 2). However, with low intensity red light (200 µmol m^{-2} s^{-1} for 48 h) and

chlorpropham, the ratio now increased, to 1.8. Red light also increased total carotenoids per cell by 22% compared to cells cultivated under white light with chlorpropham (Table 2). The overall effect of the combination of exposure to red light under conditions where cell division was arrested for 48 h therefore amounted to an increase in content of total carotenoids of 95% compared to untreated cells. These data therefore confirmed our previous work namely, that red light is able to regulate β-carotene isomerisation of *all-trans* β-carotene to *9-cis* β-carotene and will increase the *9-cis/all-trans* β-carotene ratio [22] as well as upregulate carotenoid production [21].

In the present work, red light also increased the accumulation of phytoene with the PDS inhibitor norflurazon, which was more than 2-fold the value under white light. Surprisingly, however, the content of coloured carotenoids also increased with norflurazon under red light, and β-carotene was >25% higher compared to the same treatment under white LED light. The reason for the increase is not clear. In higher plants, PDS is a plastid-localised, membrane-associated enzyme which controls flux through the carotenoid pathway by catalysing the formation of both *9,15-di-cis* phytofluene and *9,15,9′-tri-cis-ζ*-carotene from *15-cis* phytoene, in two sequential reactions and in concert with the plastidial terminal oxidase PTOX, using plastoquinone as intermediate electron acceptor and oxygen as terminal electron acceptor [4,29,36–38]. PDS has been crystallised and mechanistic details of its catalysis have been discussed [39,40]. Plastoquinone mimics, such as norflurazon, inhibit PDS by binding competitively to the plastoquinone binding site, to block phytoene desaturation [31,39,40]. Since phytoene synthesis is not blocked under these conditions, phytoene accumulates but not phytofluene or the coloured carotenoids [21,31,32]. In *D. salina*, and in contrast to [38], we recently identified the form of phytofluene in *D. salina* cultures as *9,15-di-cis* phytofluene [34]. This result demonstrates operation of a plastoquinone-dependent PDS pathway for phytofluene production in *D. salina*, like that in higher plants [40]. The increased accumulation of phytoene under red light with norflurazon is consistent with known upregulation of phytochrome-mediated synthesis of PSY transcripts in response to photo-oxidative stress [27,41,42], and is triggered by absorption of photons of red light by chlorophyll a and chlorophyll b [21,22]. Possibly red light increased not only phytoene accumulation but also the concentration of plastoquinone relative to norflurazon, to shift the competition for binding on the PDS binding site in favour of plastoquinone. This would facilitate desaturation of phytoene to β-carotene. Alternatively, there may be an additional pathway for carotenoid biosynthesis from phytoene in *D. salina*, involving a PDS which does not require plastoquinone as an electron acceptor. In non-photosynthetic bacteria and fungi, a second type of phytoene desaturase, termed CRTI, functions as an oxidase-isomerase to catalyse the complete desaturation of *15-cis*-phytoene to *all-trans*-lycopene using FAD as sole redox-active co-factor. It uses oxygen as the terminal electron acceptor, not a plastoquinone, and is not inhibited by norflurazon [39,40,43]. Further research is needed to resolve the basis for the increase in β-carotene content under red light.

Use of norflurazon under white light is known to increase photooxidation of chlorophyll and carotenes [32,35–38]. In the present work, when phytoene accumulated to similar cellular levels with norflurazon as with chlorpropham under white LED light, both coloured carotenoids and chlorophyll decreased and the net content of phytofluene declined (see Tables 1 and 2). With chlorpropham by contrast, the net content of chlorophyll increased over the same time frame (2.4-fold with 20 µM chlorpropham after 6 days, compared to untreated cells, Table 1). Phytofluene accumulated as well (see Fig. 2, Fig. 4, Table 1, Table 2 and [34]). The data with norflurazon were expected because PDS activity in *D. salina* is coupled to oxygen reduction in a chloroplastic oxygen-removing pathway as in higher plants [21,41]. Consequently, PDS-inhibition by norflurazon reduces chloroplastic oxygen reduction to water and increases the tendency for the formation of ROS along with plastid to nucleus retrograde signalling [5,29]. Phytoene accumulates but based on the number of its conjugated double bonds phytoene is still

Table 2

Pigment composition of major carotenoids and chlorophylls in *D. salina* cultures treated with 10 μM chlorpropham or 5 μM norflurazon. Cultures were maintained under white or red LED light at 200 μmol m^{-2} s^{-1} and samples were collected and analysed after 48 h treatment. Each culture condition was set up at least in triplicate ($n \geq 3$). Data presented are mean ± standard deviations. *Values estimated for total carotenoids in the table are the sum of phytoene, phytofluene, β-carotene, α-carotene, lutein and zeaxanthin. **Values estimated for total coloured carotenoids are the sum of β-carotene, α-carotene, lutein and zeaxanthin.

Carotenoids (pg cell^{-1})	T0	White LED light (48 h treatment)			Red LED light (48 h treatment)		
		No herbicide	Chlorpropham (10 μM)	Norflurazon (5 μM)	No herbicide	Chlorpropham (10 μM)	Norflurazon (5 μM)
Phytoene	0.52 ± 0.01	0.79 ± 0.04	4.41 ± 0.65	7.07 ± 0.36	0.98 ± 0.02	10.56 ± 1.53	14.42 ± 0.95
Phytofluene	0.04 ± 0.00	0.07 ± 0.00	0.67 ± 0.09	0.01 ± 0.00	0.13 ± 0.00	1.58 ± 0.17	0.02 ± 0.00
All-trans β-carotene	6.19 ± 0.11	6.46 ± 0.10	10.56 ± 1.57	4.28 ± 0.18	5.58 ± 0.60	7.91 ± 0.38	3.09 ± 0.30
9-cis β-carotene	7.92 ± 0.21	10.73 ± 0.22	13.35 ± 1.88	7.65 ± 0.67	13.40 ± 1.25	14.35 ± 1.24	11.89 ± 0.83
zeaxanthin	0.57 ± 0.01	0.90 ± 0.08	0.84 ± 0.09	0.80 ± 0.07	1.17 ± 0.12	1.05 ± 0.06	1.12 ± 0.06
All-trans α-carotene	0.33 ± 0.01	0.44 ± 0.02	0.33 ± 0.02	0.27 ± 0.01	0.41 ± 0.05	0.51 ± 0.02	0.39 ± 0.01
lutein	0.51 ± 0.02	0.66 ± 0.01	0.83 ± 0.13	0.52 ± 0.02	0.69 ± 0.04	0.74 ± 0.05	0.62 ± 0.02
*Total carotenoids	16.18 ± 0.42	20.42 ± 0.12	32.76 ± 2.02	20.27 ± 1.21	22.40 ± 0.97	39.88 ± 3.44	29.10 ± 3.19
**Total coloured carotenoids	15.62 ± 0.25	19.64 ± 0.16	29.38 ± 1.69	14.38 ± 0.46	21.17 ± 1.01	29.33 ± 1.92	17.16 ± 1.31
Chlorophyll	5.14 ± 0.31	6.46 ± 0.12	11.97 ± 1.08	5.99 ± 0.22	5.23 ± 0.41	9.15 ± 0.73	4.96 ± 0.13
Ratio total carotenoids: chlorophyll	3.2	3.2	2.7	3.4	4.3	4.4	5.9
Ratio β-carotene: chlorophyll	2.7	2.7	2.0	2.0	3.6	2.4	3.0
Ratio phytoene: coloured carotenoids	0.03	0.04	0.15	0.49	0.05	0.36	0.84
Ratio *9cis* β-carotene: *all-trans* β-carotene	1.3	1.7	1.3	1.8	2.4	1.8	3.8

less effective as an antiradical scavenger than β-carotene, albeit having a higher antioxidant activity than expected [8, and refs therein]. Consequently, ROS accumulate and the net chlorophyll concentration declines.

The data with chlorpropham cannot be reconciled in terms of a stress response caused by N-starvation (see Fig. 4A). They do however indicate breakdown in the carotenoid biosynthetic pathway that hinders the normal conversion from phytoene to coloured carotenoids in the chloroplast at the level of PDS functionality, which is independent of chlorophyll biosynthesis and involves division of the cell nucleus.

The simplest explanation to rationalise these data recognises that both phytoene and chlorophyll are synthesised from a common GGPP precursor [4] and their biosynthetic pathways are under phytochrome control as in higher plants [21,44]. In the case of carotenoids, carotenogenic enzymes, many of which appear to be localised within membranes, may be clustered in metabolic channels or 'metabolons' for activity [45]. When chlorpropham inhibited nuclear cell division (Fig. 2), it also impaired further recruitment of carotenogenic biosynthetic enzymes into biologically-active, membrane-located metabolons. This would explain why, with chlorpropham, red light increased the content of phytoene compared to under white light but failed to increase the content of β-carotene (Table 2) and also why the *9-cis/all trans* β-carotene ratio with chlorpropham was less responsive to red light effects compared to either cells treated in the absence of herbicide, or treated with norflurazon. PDS functionality on flux through the carotenoid biosynthetic pathway from phytoene to β-carotene requires to be membrane-associated for the redox reactions involved [21,26]. Davidi et al. [15] proposed that in *D. salina* the lipids required for formation of the carotenoid-containing βC-plastoglobuli were derived, in part, from (nuclear-encoded) cytoplasmic lipid droplets (CLDs) that formed in the endoplasmic reticulum and coalesced with chloroplast envelope membranes and in part, from the hydrolysis of chloroplast membrane lipids. Little is known about co-ordination between these pathways or about where the enzymes of the carotenoid pathway are localised. However, in *D. salina* treated with norflurazon, accumulating phytoene is sequestered into plastoglobuli in the inter-thylakoid space of the chloroplast [37]. With chlorpropham, accumulating phytoene may also be sequestered into plastoglobuli when recruitment of carotenogenic enzymes into biologically-active, membrane-located metabolons, fails. This needs

to be determined.

5. Conclusions

Cultivation of *D. salina* with the carbamate herbicide, chlorpropham, which is widely used as a sprout inhibitor, arrested cell division and the colourless carotenoid phytoene accumulated massively, along with phytofluene and, to a much lesser extent, the coloured carotenoids. The chlorophyll content also increased. The data confirmed that the amount of phytoene or β-carotene that accumulates in *D. salina* is positively correlated with the integral irradiance to which the cells are exposed during a division cycle. Cultivation under red light was shown to be more effective than white light in increasing total carotenoids per cell. We suggest that treatment with chlorpropham disrupted synchronised control between nuclear and chloroplast events and recruitment of carotenogenic enzymes into biologically active, membrane-located metabolons. Phytoene and phytofluene are colourless carotenoids which are sought after for the development of nutricosmetics and other health/beauty products. The production of phytoene and phytofluene can be improved by increasing the cell density at the outset of chlorpropham treatment, and with high light intensity and use of red wavelength light. The results lay the basis for a new method with low toxicity for production of colourless carotenoids based on the cultivation of *D. salina* [46].

Author contributions

Conceptualization, P·H; methodology, Y.X.; formal analysis, P·H.; Y.X.; data curation, Y.X.; writing—original draft preparation, P·H; writing—review and editing, P.H; Y.X.; visualization, P.H.; Y.X; supervision, P.H.; project management, P.H.

Declaration of competing interest

The authors declare that they have no known competingfinancialinterests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2020.102126.

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