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1 *Article*

Red light control of β-carotene isomerisation to *9-cis* β-carotene and carotenoid accumulation in *Dunaliella salina*

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8

9 **Abstract:** *Dunaliella salina* is a rich source of *9-cis* β-carotene, which has been identified as important
10 in the treatment of retinal dystrophies and other diseases. We previously showed that chlorophyll
11 absorption of red light photons in *D. salina* is coupled to oxygen reduction and phytoene
12 desaturation and increases the pool size of β-carotene [1]. Here we show for the first time that
13 growth under red light also controls conversion of extant *all-trans* β-carotene to *9-cis* β-carotene by
14 β-carotene isomerases. Cells illuminated with red light from a light emitting diode (LED) during
15 cultivation contained a higher *9-cis* β-carotene content compared to cells illuminated with white or
16 blue LED light. The *9-cis/all-trans* β-carotene ratio in red light treated cultures reached >2.5:1 within
17 48 hours and was independent of light intensity. Illumination using red light filters that eliminated
18 blue wavelength light also increased the *9-cis/all-trans* β-carotene ratio. With norflurazon, a
19 phytoene desaturase inhibitor which blocked downstream biosynthesis of β-carotene, extant
20 *all-trans* β-carotene was converted to *9-cis* β-carotene during growth with red light and the
21 *9-cis/all-trans* β-carotene ratio was ~2:1. With blue light under the same conditions, *9-cis* β-carotene
22 was likely destroyed at a greater rate than *all-trans* β-carotene (*9-cis/all-trans* ratio 0.5:1). Red light
23 perception by the red light photoreceptor, phytochrome, may increase the pool size of anti-oxidant,
24 specifically *9-cis* β-carotene, both by upregulating phytoene synthase to increase the rate of
25 biosynthesis of β-carotene and to reduce the rate of formation of reactive oxygen species (ROS), and
26 by upregulating β-carotene isomerases to convert extant *all-trans* β-carotene to *9-cis* β-carotene.

27 **Keywords:** *9-cis* β-carotene; *all-trans* β-carotene; *Dunaliella salina*; red LED; blue LED; growth; light
28 intensity; carotenoids; isomerisation
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30 1. Introduction

31 Carotenoids are orange, yellow or red pigments which are synthesized by all photosynthetic
32 organisms for light-harvesting and for photo-protection, and stabilise the pigment-protein
33 light-harvesting complexes and photosynthetic reaction centres in the thylakoid membrane [1-4].
34 *Dunaliella salina*, a halotolerant chlorophyte, is one of the richest sources of natural carotenoids and
35 accumulates up to 10 % of the dry biomass as β-carotene under conditions that are sub-optimal for
36 growth i.e. high light intensity, sub-optimal temperatures, nutrient limitation and high salt
37 concentrations [5-8]. Two pools of β-carotene have been identified, which may be distinguished on
38 the basis of geometric isomer configuration, *cis* or *trans* (Z/E), and enzyme complement. Thylakoid
39 β-carotene consists principally of *all-trans* β-carotene (*all-trans* βC), and may be constitutively
40 expressed; the 'accumulated' β-carotene, which is found in globules of lipid and proline-rich,
41 β-carotene globule protein (the βC-plastoglobuli) in the inter-thylakoid spaces of the chloroplast,
42 appears in high concentration of both *cis/trans* (*Z/E*) configurations, ~1:1 [5,9-11].
43 The occurrence of such high concentrations of *9-cis* βC in *D. salina* is of great pharmaceutical
44 interest. *9-cis* βC has a higher antioxidant activity than *all-trans* βC, and may also be more efficient

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than *all-trans* βC *in vivo* [12,13]. *9-cis* βC has been proposed in treatments for retinal dystrophies, chronic plaque psoriasis and atherosclerosis and as an anti-ageing therapy [14-18]. Importantly, a synthetic pure preparation of *9-cis* βC has recently been shown to inhibit photoreceptor degeneration of eye cups from mice with a retinoid cycle genetic defect [19].

However the mechanism and regulation of the biosynthesis of *9-cis* βC in *D. salina* is unclear. Using different inhibitors of β-carotene biosynthesis, Shaish *et al* [20] found that all the intermediates between phytoene and β-carotene in cultures maintained under low light intensity and N-starvation contained similar ratios of *9-cis/all-trans* stereoisomers. They concluded that the isomerisation step must occur at or before phytoene, and that no further isomerisation was likely to occur during the further transformation of phytoene to β-carotene. On the other hand in cultures maintained under light stress, *9-cis/all-trans* βC isomerases were identified in high concentrations in plastidic globules and shown *in vitro* to catalyse conversion of *all-trans* βC to *9-cis* βC, whilst expression of the corresponding genes was enhanced under stress conditions [21]. The *9-cis/all-trans* βC ratio has been shown to increase four-fold and the β-carotene content two-fold when the culture temperature decreased from 30 °C to 10 °C [22], and to increase with increased light intensity [21,23,24], but to be independent of light wavelength within the photosynthetically active range [7]. There have also been reports of a higher *9-cis/all-trans* βC ratio in *D. salina* cultivated under low light intensities [25,26].

Recently we showed that growth of *D. salina* under high intensity red light was associated with carotenoid accumulation and a high rate of oxygen uptake [1]. We proposed a mechanism for carotenoid synthesis under red light, which involved absorption of red light photons by chlorophyll to reduce plastoquinone in photosystem II, coupled to phytoene desaturation by a plastoquinol:oxygen oxidoreductase, with oxygen as electron acceptor. Partitioning of electrons between photosynthesis and carotenoid biosynthesis would depend on both red photon flux intensity and phytoene synthase upregulation by the red light photoreceptor, phytochrome.

In this paper the effects of red and blue light on the β-carotene isomeric composition in *D. salina* were investigated. Isomerisation between *all-trans* and *9-cis* βC in *D. salina* was regulated by light wavelength but not light intensity, with red light shifting the equilibrium in the direction of *9-cis* βC production. In blue light, *9-cis* βC was more rapidly destroyed than *all-trans* βC.

2. Materials and Methods

2.1. Strains and cultivation

D. salina strain CCAP 19/41 (PLY DF15) was isolated from a salt pond in Israel and obtained from the Marine Biological Association, UK (MBA). Algae were cultured in Modified Johnsons Medium [27] containing 1.5 M NaCl, which has been tested as the optimal salinity for cell growth of the strain. The algae were cultured in Erlenmeyer flasks (Fisher Scientific, UK) containing 500 mL culture each in an ALGEM Environmental Modeling Labscale Photobioreactor (Algenuity, Bedfordshire, UK) at 25 °C with white, red or blue LED lights provided from the bottom of the bioreactor chamber. Red filters (Lee filter 26 Bright red, 27 Medium red, and 787 Marius red) when used, were purchased from Lee Filters Andover (Hampshire, UK) and placed over the LED lights.. The cultures were shaken for 10 min at 100 rpm every hour before taking samples to monitor cell growth. Cell density of cultures was determined by counting the cell number of cultures using a haemocytometer after fixing with 2 % formalin.

2.2. Carotenoids analysis

The composition of pigments was analysed by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) (Agilent Technologies 1200 series, Agilent, Santa Clara, United States), using a YMC30 250 × 4.9 mm I.D S-5μ HPLC column (YMC, Europe GmbH) at 25 °C with an isocratic solvent system of 80 % methanol: 20 % methyl tert-butyl ether (MTBE) and flow rate of 1 mL min^{-1} at a pressure of 90 bar. A carotene standard for *all-trans* βC was obtained from

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Sigma-Aldrich Inc. (Merck KGaA, Darmstadt, Germany); a carotene standard for *9-cis* βC was obtained from Dynamic extraction (UK). Standards were dissolved in methanol to generate standard curves and DAD scans analysed at wavelengths of 280 nm (phytoene), 355 nm (phytofluene), 450 nm (β-carotene, α-carotene, lutein and zeaxanthin), and 663 nm (chlorophylls). The *all-trans* and *9-cis* βC contents were quantified from their absorption at 450 nm.

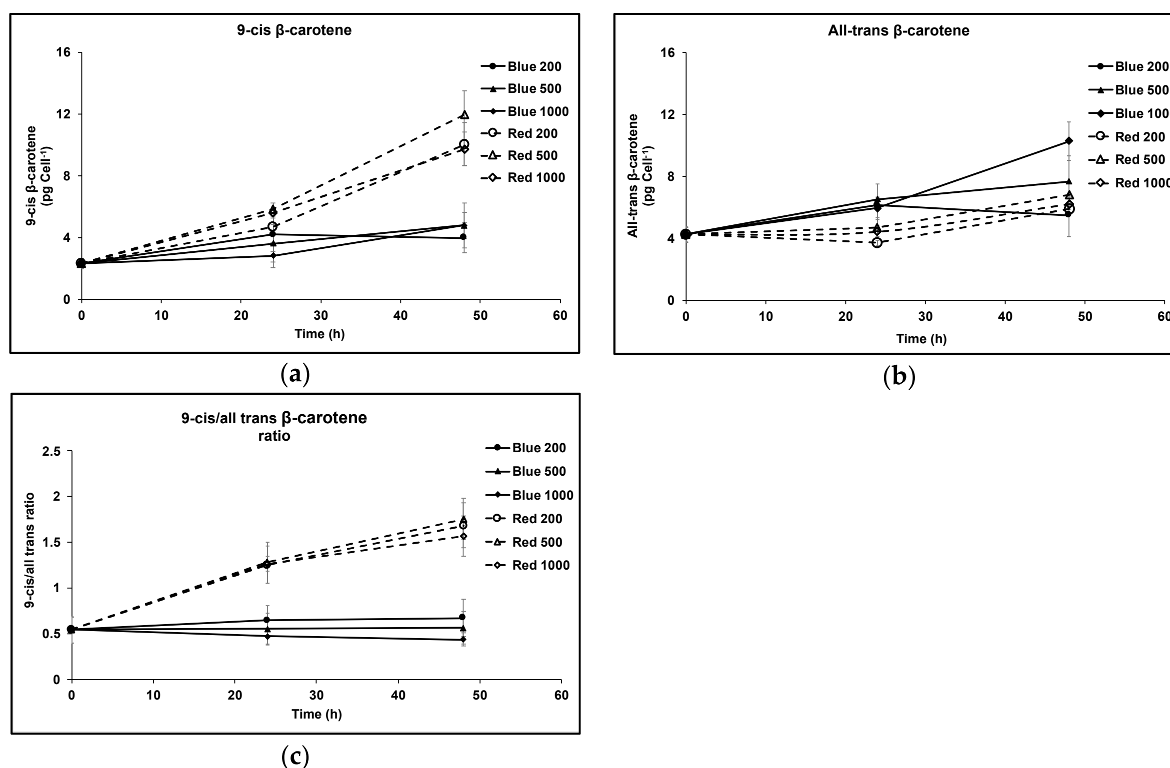
Samples of culture (3 mL) were harvested by centrifugation at 3000× *g* for 10 min and pigments extracted after sonication for 20 s with 2 mL MTBE–MeOH (20:80). Samples were clarified at the centrifuge then filtered (0.45 µm filter) into amber HPLC vials before analysis.

2.3. Statistical analysis

Each experiment was carried out at least in triplicate. Data collected were analyzed in R by one way ANOVA with posterior Dunnett's test and Turkey multiple pairwise-comparisons. A $p<0.05$ value was considered significant.

3. Results

9-*cis* βC and *all-trans* βC were the major carotenoids that accumulated in *D. salina* biomass after 48 hours exposure to red or blue LED light, but the relative pool sizes of each depended on the concentration of red and blue photons of light received. Under blue light, the contents of both *9-cis-* and *all-trans* βC per cell increased with time (Figure 1 a, b), and the ratio of *cis/trans* βC isomers remained approximately the same at all light intensities (Figure 1 c). The concentration of *9-cis* βC was ~half as much as *all-trans* βC. Under red light by contrast, the concentration of *9-cis* βC and total pool of carotenoids increased massively compared to that in blue in all light intensities and the content of *9-cis* βC was ~twice as much as *all-trans* βC (Figure 1 a, b). With increasing light intensity the relative pool sizes of the isomers changed; that of *all-trans* βC decreased and that of *9-cis* βC increased. Furthermore *9-cis* βC increased with time to > 60 % of total β-carotene under red light (Figure 1 d). HPLC profiles of the carotenoid extracts showed 9-*cis* βC and *all-trans* βC were the major carotenoids that accumulated in *D. salina* biomass, and that the ratios of the two isomers were different under different wavelengths (Figure 1 e).



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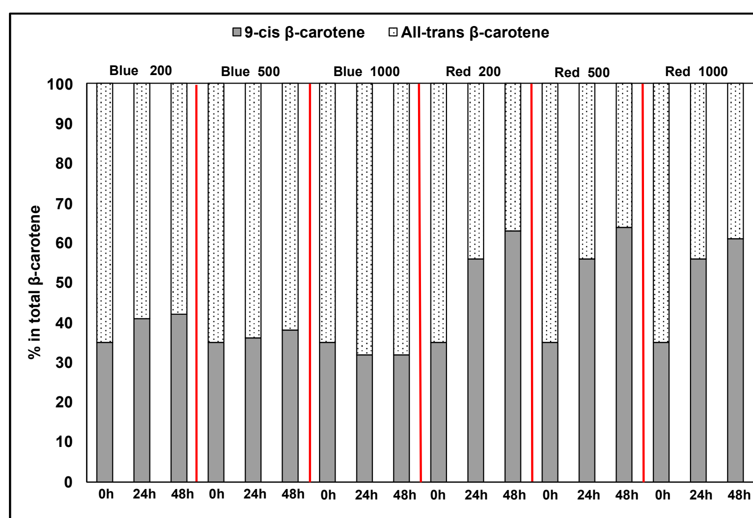
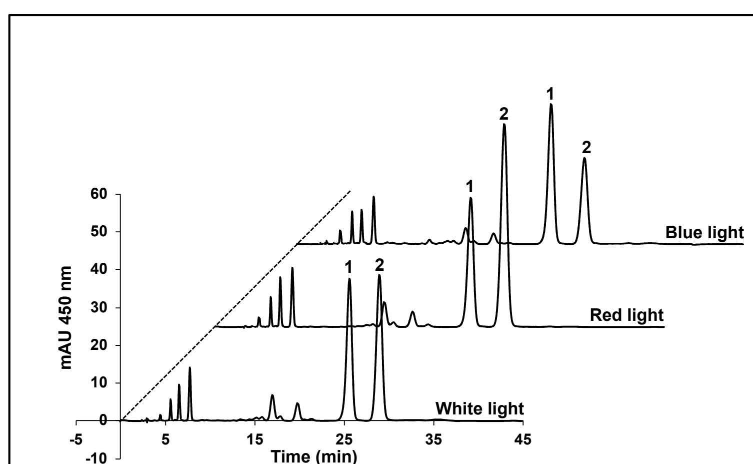


(**d**)



(**e**)

Figure 1. Cultivation of *D. salina* under continuous blue or red LED light at three different light intensities of 200, 500 and 1000 µmol m^{-2} s^{-1} for 48 hours. (**a**) Cellular content of *9-cis* βC, (**b**) cellular content of *all-trans* βC; (**c**) *9-cis/all-trans* βC. (d) Percentage of *9-cis* and *all-trans* βC in total βC . *D. salina* cells were grown under 12/12 light/dark (L/D) with 200 µmol m^{-2} s^{-1} supplied by white LED light to exponential growth phase, then dark-adapted for 36 hours. After dark adaptation, they were transferred to continuous white, blue or red LED light at light intensities of 200, 500, or 1000 µmol photons m^{-2} s^{-1} for 48 hours. Samples were taken at 0, 24 and 48 hours for carotenoids analysis. (**e**) HPLC profiles at 450 nm of carotenoid extracts from *D. salina* cultivated under continuous white light, red light or blue light, each at 1000 µmol m^{-2} s^{-1} for 48 hours. Peak 1: *all-trans* β-carotene; peak 2: *9-cis* β-carotene. Biomass was collected at 48 hours illumination and carotenoids extracted for HPLC analysis. Each culture condition was set up at least in triplicate.

To test the effect of blue light exposure on carotene isomers that had accumulated in red light and vice versa, dark-adapted cultures of *D. salina* were cultivated in red or blue LED high intensity light for 24 hours (T0), and then cultivated for a further 24 hours in red, blue, or a mixture of red and blue LED light (1:1) with the same light intensity, or the dark. As before, red-acclimated cells maintained in red light produced the greatest amount of carotenoids with ~twice as much as *9-cis* βC as *all-trans* βC (Figure 2). On the other hand *9-cis* βC decreased when red-acclimated cells were transferred to blue light (Figure 2), to the same level as for blue-acclimated cells maintained continuously in blue (Figure 3); the pool size of carotenoids for both conditions was about the same and the concentration of *9-cis* βC was ~half as much as *all-trans* βC. Conversely, blue-acclimated cells

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140 when transferred to red LED produced more carotenoids (28 % greater content), principally as *9-cis*
141 βC (Figure 3).
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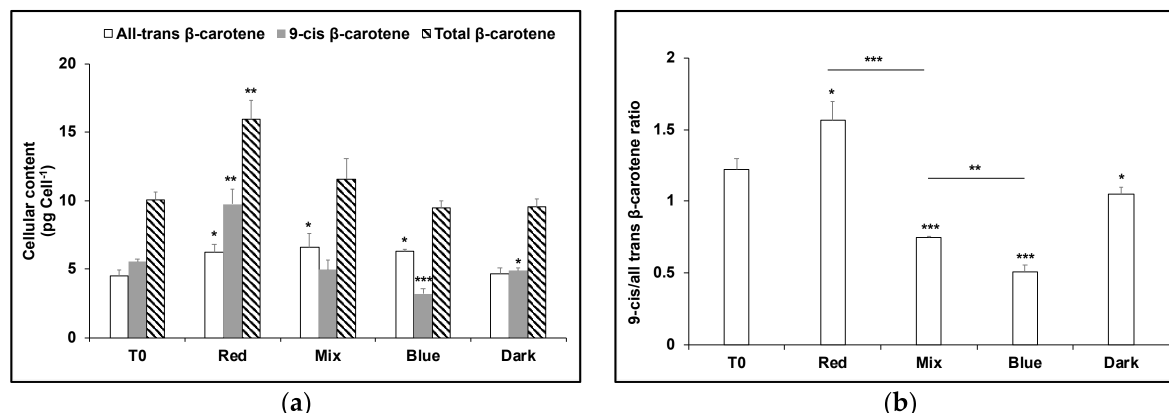

143 **Figure 2.** (**a**) Cellular content of *9-cis* βC and *all-trans* βC and (**b**) *9-cis/all-trans* βC ratio in *D. salina*
144 cultures exposed to continuous red LED light at 1000 μmol m^{-2} s^{-1} for 24 hours followed by 24 hours
145 under either red light, a mix of 1:1 red and blue light, blue light at the same light intensity of 1000
146 μmol m^{-2} s^{-1} or dark. Each culture condition was set up at least in triplicate. Results were analysed by
147 one way ANOVA with posterior Dunnett's test compared to T0 and Tukey multiple
148 pairwise-comparisons. Asterisks represent different levels of significance (***$0<p\leq0.001$,
149 **$0.001<p\leq0.01$, *$0.01<p\leq0.05$).

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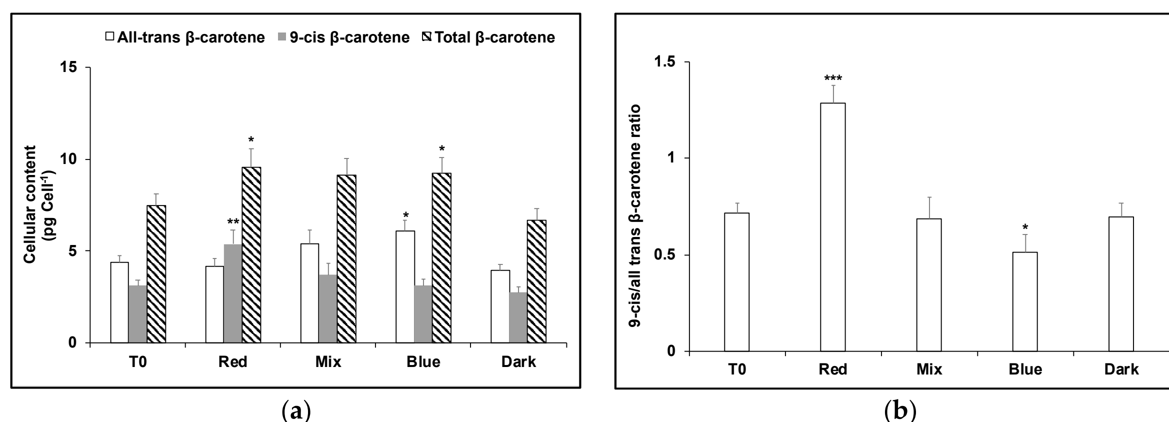

151 **Figure 3.** (**a**) Cellular content of *9-cis* βC and *all-trans* βC and (**b**) *9-cis/all-trans* βC ratio in *D. salina*
152 cultures exposed to continuous blue LED light at 1000 μmol m^{-2} s^{-1} for 24 hours followed by 24 hours
153 under either red light, a mix of 1:1 red and blue light, blue light at the same light intensity of 1000
154 μmol m^{-2} s^{-1} or dark. Each culture condition was set up at least in triplicate. Results were analysed by
155 one way ANOVA with posterior Dunnett's test compared to T0 and Tukey multiple
156 pairwise-comparisons. Asterisks represent different levels of significance (***$0<p\leq0.001$,
157 **$0.001<p\leq0.01$, *$0.01<p\leq0.05$).

158 Since red light increased the net content of *9-cis* βC, the effects of red light/dark cycles of
159 increasing red light duration during cultivation were tested. Increasing red light duration increased
160 the total amount of β-carotene, in particular the amount of *9-cis* βC (Figure 4). With a red light/dark
161 cycle of 10 min/110 min, the ratio of *9-cis/all-trans* βC was 1.1:1, but in a red light/dark cycle of 30
162 min/30 min, this increased to 2.2:1, similar to that in continuous red (2.3:1). However in continuous
163 red light, the total pool size β-carotene was nearly 25 % greater.
164

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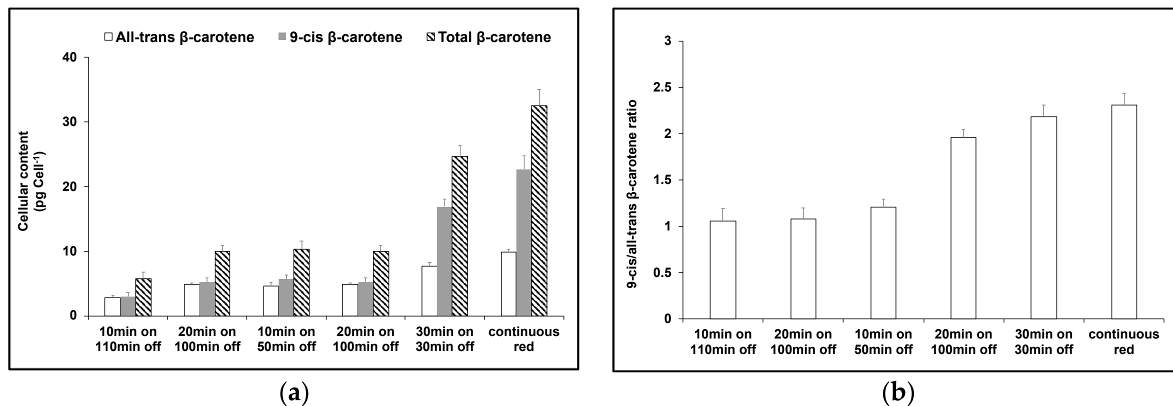


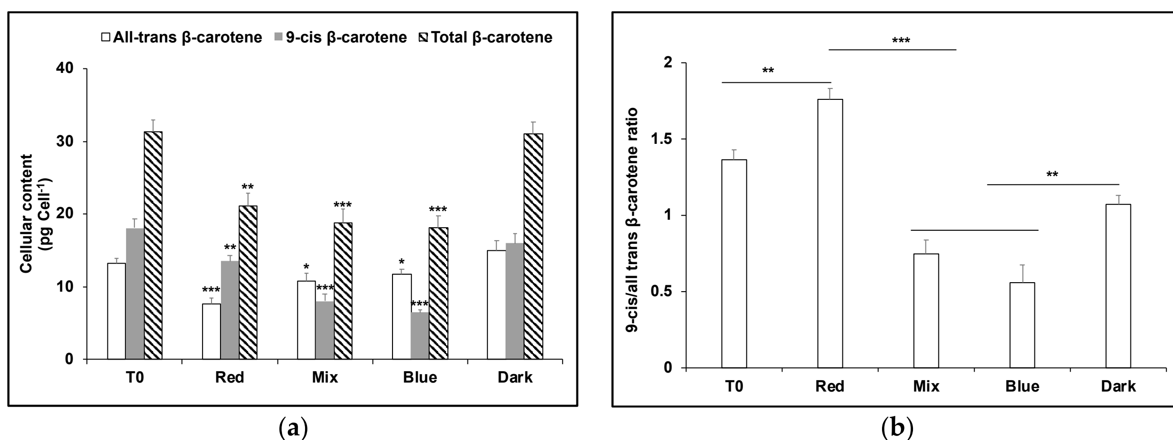
(**a**) (**b**)

165 **Figure 4.** Effect of cultivating D. salina under different red light/dark cycles. (**a**) Cellular content of *9-cis*
166 βC, *all-trans* βC and total βC and (**b**) *9-cis/all-trans* βC ratio of *D. salina* cultures grown under different light/dark
167 cycles of red LED light supplied at 500 μmol m^{-2} s^{-1} . Cultures of *D. salina* were grown to a cell density of ~ 0.2
168 million cells mL^{-1} under white LED light and then transferred into red LED light growth cycles of different
169 duration, which were maintained for 6 days.

170

171 Accumulation of carotenoids under red light has previously been shown to involve
172 upregulation of phytoene synthase to increase the pool size of phytoene in *D. salina* cultures [1]. In
173 order to test the effect of blue and red light on the β-carotene isomer composition, but without
174 interference of *de novo* synthesis of β-carotene from phytoene, norflurazon, a phytoene desaturase
175 inhibitor, was applied to the *D. salina* cultures (Figure 5). After 48 hours without light, the total pool
176 size of carotenoids was the same as that at the outset of the experiment (T0) before light treatment i.e.
177 norflurazon blocked any further downstream synthesis of β-carotene. Under these conditions, the
178 β-carotene isomer composition, *9-cis/all-trans* βC, was 1.1:1, the same as that recorded for growth in a
179 red light/dark cycle of 10 min/110 min. Both red and blue light treatments lowered the total pool size
180 of total β-carotene, blue more than red: ~ 31-32 % total β-carotene was destroyed under red light and
181 under the 1:1 red/ blue light mix, and ~41 % under blue light. Carotenoids absorb photons in the
182 range 400-550 nm, exactly overlapping the emission spectrum of the blue LED (440-500 nm)
183 therefore the greater loss in blue light compared to red was to be anticipated. Furthermore, although
184 both *all-trans* βC and *9-cis* βC were destroyed under blue light, the loss of *9-cis* βC was very much
185 greater: only ~40 % of the content of *9-cis* βC recorded in dark-treated cultures remained, compared
186 to 78 % for *all-trans* βC. Since *9-cis* βC has a higher antioxidant activity than *all-trans* βC, this result
187 might also be anticipated. Somewhat surprisingly, however, loss of *9-cis* βC under red light
188 compared to blue was much smaller and the ratio of *9-cis/all-trans* βC was 3-fold greater than under
189 blue light. Since the emission spectrum of the red LED (625-680 nm) emits photons that are not
190 absorbed by β-carotene, these data imply isomerisation of extant *all-trans* βC to *9-cis* βC to increase
191 the content of *9-cis* βC at the expense of *all-trans* βC during growth.

192



(**a**) (**b**)

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Figure 5. Production of carotenes in *D. salina* cultures treated with 5 µM norflurazon. (**a**) cellular content of *9-cis* βC, *all-trans* βC and total βC and (**b**) *9-cis/all-trans* βC ratio. Cultures were grown for 24 h under white LED light then treated with norflurazon and maintained for a further 48 h under red, blue or a mix of red and blue LED light at 200 µmol m^{-2} s^{-1} or kept in the dark. T0: time point after growth for 24h under white LED light only, before addition of norflurazon. Results were analysed by one way ANOVA with posterior Dunnett's test compared to T0 and Tukey multiple pairwise-comparisons. Asterisks represent different levels of significance (***0<p≤0.001, **0.001<p≤0.01, *0.01<p≤0.05).

A similarly greater loss of *all-trans* βC compared to *9-cis* βC in red light was obtained using Lee Bright Red, Medium Red or 787 Marius Red filters: these transmitted only a fraction (8.6 %, 3.6 % and 1.0 %) of the light intensity applied with a red LED (1000 µmol m^{-2} s^{-1}), but importantly excluded light wavelengths below 550 nm. Each increased the total β-carotene pool size and the *9-cis/all-trans* βC ratio was higher (Figure 6). With the 787 Marius Red filter, cells received only approximately 10-17 µmol m^{-2} s^{-1} light intensity of the red wavelength but this was still sufficient to increase the ratio of *9-cis/all-trans* βC ratio, the amount of *9-cis* βC per cell and total β-carotene to values approaching those found using white light at 1000 µmol m^{-2} s^{-1}.

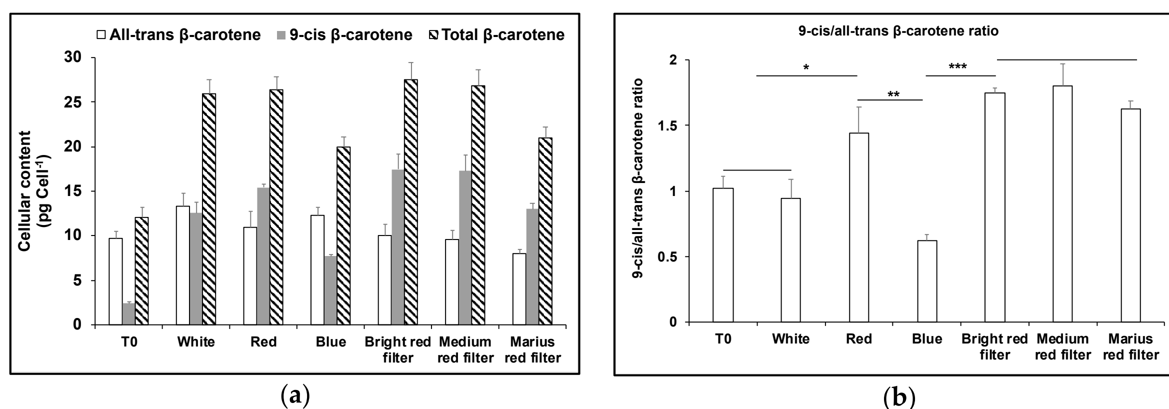

Figure 6. Cultivation of *D. salina* using red light filters. *D. salina* was cultivated under white light to early orange phase (cell density of ~0.5 x 10^6 cells mL^{-1}; carotenoid: chlorophyll ratio ~3), and then cultures were diluted with fresh medium to a cell density of ~0.2 x 10^6 cells mL^{-1} (no nutrient stress) (T0) and then further cultivated for 48 hours under white, red or blue LED light at 1000 µmol m^{-2} s^{-1} or under white LED light at 1000 µmol m^{-2} s^{-1} covered with one of three different red filters (Lee filter 26 Bright red; Lee filter 27 Medium red; or Lee filter 787 Marius red). (**a**) Cellular content of *9-cis*, *all-trans* and total β-carotene. (**b**) *9-cis/all-trans* β-carotene ratio. Results were analysed by one way ANOVA and Tukey multiple pairwise-comparisons. Asterisks represent different levels of significance (***0<p≤0.001, **0.001<p≤0.01, *0.01<p≤0.05).

The co-regulation by light and temperature on the β-carotene production and isomeric composition in *D. salina* is shown in Figure 7. Cultivation at 15 °C compared to 25 °C increased the *9-cis/all-trans* βC ratio, especially under red light, but decreased the pool size of β-carotene measured over the same time frame (48 h).

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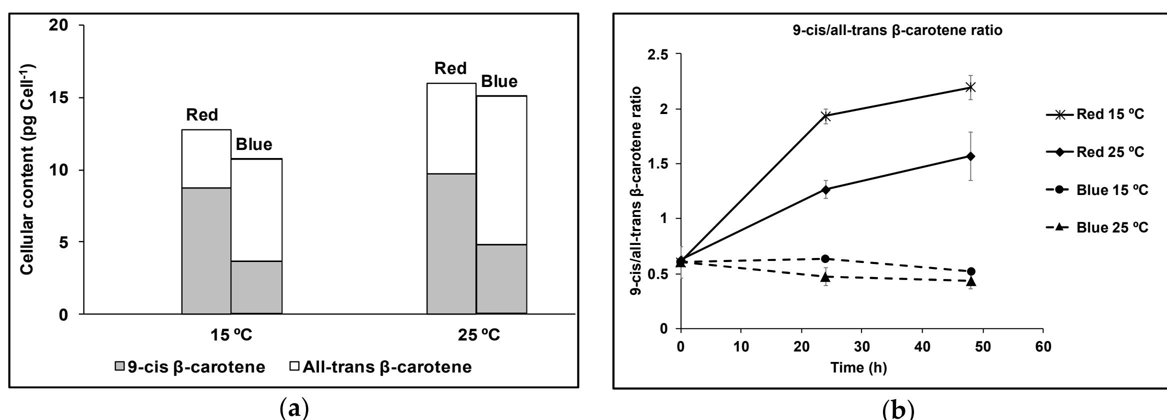

(a) (b)

224 **Figure 7.** *D. salina* cultivated under red or blue light at either 15 °C or 25 °C. (**a**) Cellular content of *9-cis*
225 β-carotene and *all-trans* β-carotene (**b**) *9-cis/all-trans* β-carotene ratio. Cells were cultured under a light:dark
226 12h:12h white light growth regime to mid-log phase of the growth cycle (0.1-0.2 x 10^6 cells mL^{-1}) then transferred
227 to the dark for 24 h before treatment for 48 hours at either 15 °C or 25 °C under continuous blue or red LED light
228 at 1000 μmol m^{-2} s^{-1}. Each culture condition was set up at least in triplicate.
229
230 Finally the effects of blue and red light on the destruction of *all-trans* βC were evaluated. No
231 reaction of *all-trans* βC solutions was detected under red light in nitrogen (Figure 8 a). Under red
232 light in air, (Figure 8 b), 40 % destruction of *all-trans* βC was recorded, whereas in blue light (Figure 8
233 c), *all-trans* βC was fully destroyed within the same time frame. These data show that blue light is
234 more damaging to *all-trans* βC than is red light.
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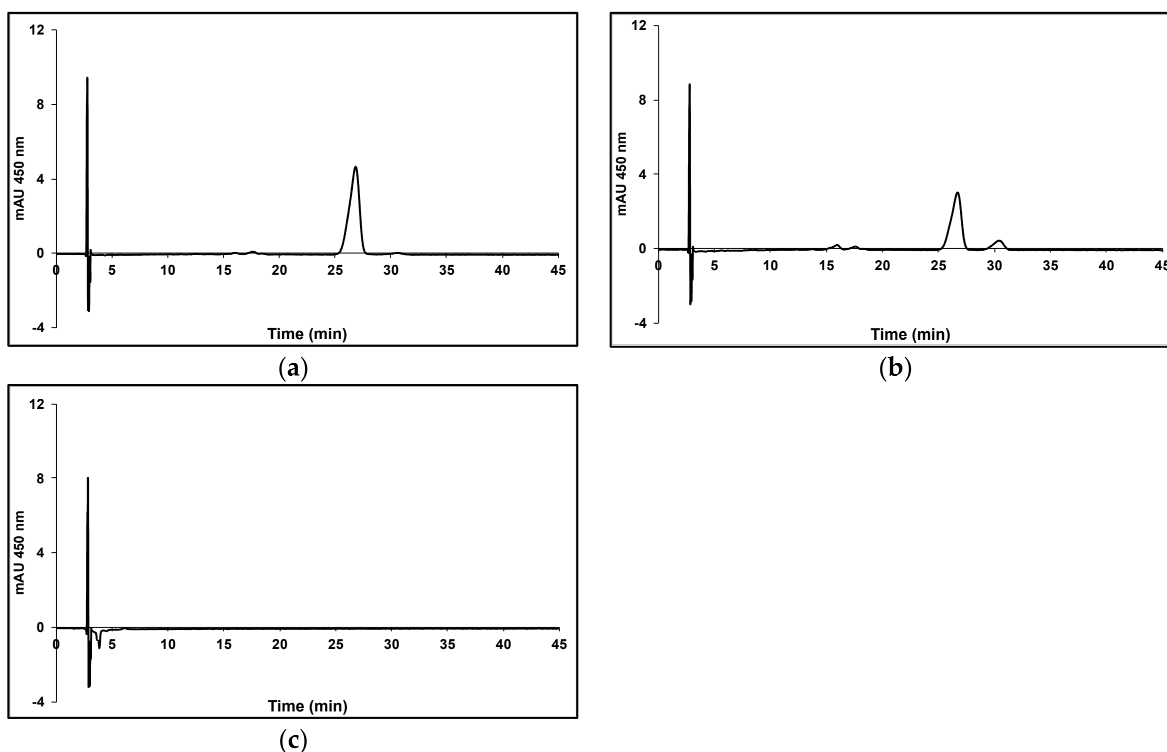

(a) (b) (c)

236 **Figure 8.** Effect of red or blue LED light on the photo-destruction of *all-trans* βC. *All-trans* βC was
237 dissolved in chloroform to a final concentration of 2.4 μM and vials were thoroughly flushed with
238 either nitrogen or air, sealed and incubated for 24 h at 25 °C under different LED lights at 200 μmol
239 m^{-2} s^{-1}. (**a**) Red light under nitrogen; (**b**) Red light in air; (**c**) Blue light in air.

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241 **4. Discussion**

242 In the present work we found that under high intensity red LED light (up to 1000 μmol m^{-2} s^{-1})
243 but in conditions of nutrient sufficiency, *D. salina* accumulated carotenoids rapidly within 48 hours.
244 Surprisingly, the major accumulated isomer was *9-cis* βC, ~twice as much as *all-trans* βC. In vitro,
245 *9-cis* βC is a better scavenger of free radicals than *all-trans* βC [12] and reportedly degrades more
246 rapidly compared to *all-trans* βC under both light and dark conditions [28]. Furthermore chlorophyll
247 absorbs photons in the range of the emission spectrum of the red LED used here (625-680 nm) and
248 therefore in *D. salina* cultures in high intensity red light, a high rate of photo-oxidation of *9-cis* βC
249 might have been anticipated. Carotenoids are known antioxidants synthesized by many microalgae
250 to prevent photoinhibition caused by photo-oxidation of photosynthetic reaction centres.
251 Photooxidative damage occurs when species such as singlet oxygen ($^1O_2$) are formed under
252 saturating light conditions as a result of transfer of energy from chlorophyll in the triplet excited
253 state ($^3Chl^*$) to the ground state of O_2. $^1O_2$ react readily with fatty acids to form lipid peroxides and
254 will set up a chain of oxygen activation events that may eventually lead to a hyperoxidant state and
255 cell death [29]. Carotenoids protect the photosystems by reacting with lipid peroxidation products
256 and terminating free radical chain reactions as a result of the presence of the polyene chain; by
257 scavenging $^1O_2$ and dissipating the energy as heat; by reacting with triplet excited chlorophyll $^3Chl^*$
258 to prevent formation of $^1O_2$ or by dissipation of excess excitation energy through the xanthophyll
259 cycle [3,30,31].

260 The simplest explanation to resolve the seeming anomaly, namely accumulation of the more
261 readily degraded *9-cis* βC under high intensity red light conditions that should be associated with
262 high rates of photo-oxidation, invokes the activity of β-carotene isomerases, the gene transcripts of
263 which are increased in light stress [21]. Davidi *et al*. [11] showed that all the enzymes in the
264 biosynthetic pathway from phytoene to β-carotene were present in the plastidic lipid globules and
265 included enriched concentrations β-carotene isomerases; two of these, *9-cis*-βC-ISO*1*
266 and *9-cis*-βC-ISO*2*, were shown to be responsible for the catalytic conversion of *all-trans* βC to *9-cis*
267 βC. Based on the data presented here we propose that the expression of gene transcripts of
268 β-carotene isomerases may be triggered by specific light sensing, possibly through phytochrome.

269 In red light compared to blue, the apparent loss of *9-cis* βC with norflurazon was surprisingly
270 small and the ratio of *9-cis/all-trans* βC was 3-fold greater than in blue light (Figure 5). Accumulation
271 of *9-cis* βC by phytoene synthase (PSY) gene activation, whose expression has been shown to be
272 greatly increased 6-48 hours following stress [11] was precluded by the presence of norflurazon,
273 which blocked phytoene desaturation and consequent carotene synthesis. Under these conditions,
274 the relative increase in pool size of *9-cis* βC in red light implies a much higher rate of *9-cis* βC
275 formation from extant *all-trans* βC, caused by increased isomerase activity, than the rate of *9-cis* βC
276 destruction (see Figure 5). Carotenes absorb photons in the range 400-550 nm, exactly overlapping
277 the emission spectrum of the blue LED (440-500 nm). However blue light catalysed a much more
278 rapid rate of destruction of carotenes than red light (Figure 8). In blue LED light, *9-cis* βC would be
279 destroyed more rapidly than could be replenished by adjustment of the *9-cis/all-trans* βC equilibrium
280 position because increased β-carotene isomerase activity from red-light activated gene expression
281 for β-carotene isomerases is not possible in blue light (see Figure 5).

282 Red light stimulation of the expression of gene transcripts of β-carotene isomerases by a
283 phytochrome to increase the rate of accumulation of *9-cis* βC by β-carotene isomerases is also
284 supported by the increase in pool size of *9-cis* βC under low intensity red light (Figure 6). Each of the
285 Lee red light filters increased the total β-carotene pool size and the *9-cis/all-trans* βC ratio was higher
286 despite the much lower light intensity of the red wavelength compared to the red LED light. The
287 effects of low temperature on *9-cis* βC-accumulation in *D. salina* are also noteworthy, since enzyme
288 catalysis typically shows a Q_{10}~2, yet in the present work, formation of *9-cis* βC in low temperature
289 compared to high was increased under red light, and had little effect in blue. In higher plants, the
290 activated phytochrome B, a red light photoreceptor, is considered to function as the thermal sensor
291 to sense environmental temperature [32]. Mutants with no phytochromes showed a constitutive
292 warm temperature transcriptome even at low temperatures [33]. Red light sensing to increase the

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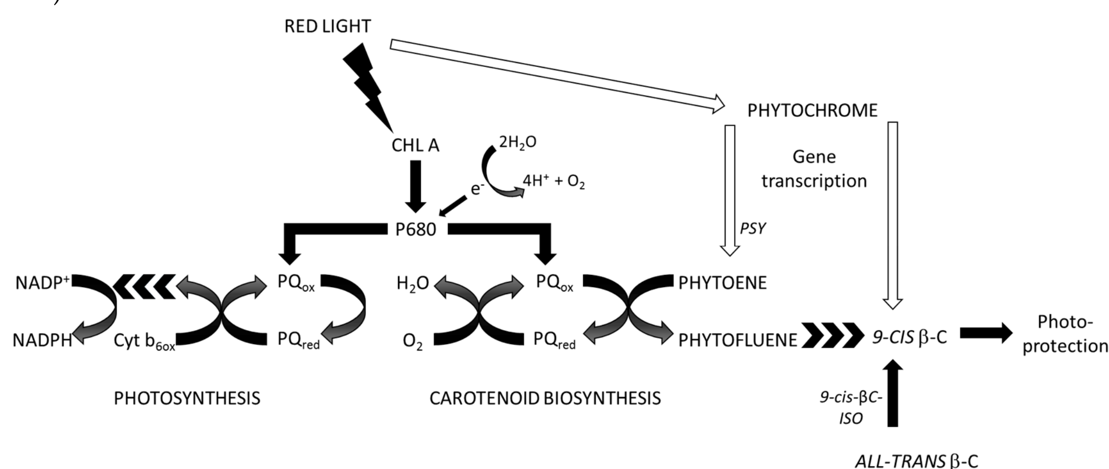
concentration of β-carotene isomerases and catalyse conversion of *all-trans* βC at low temperatures as well as high may play a significant role in photoprotection in *D. salina*.

We recently proposed that red light enhanced the production of carotenoids in a mechanism dependent on both photon flux intensity as well as upregulation of phytoene synthase by the red light photoreceptor phytochrome and that chlorophyll absorption of red light photons and subsequent plastoquinone reduction in photosystem II was coupled to oxygen reduction and phytoene desaturation by plastoquinol:oxygen oxidoreductase [1]. Red light sensing by phytochrome to increase the pool size of phytoene by phytoene synthase has been reported in higher plants [34]. Red light control of carotenoid biosynthesis coupled to accumulation of the more readily oxidized *9-cis* βC as a consequence of isomerisation from *all-trans* βC reserves would therefore rapidly increase the pool size of anti-oxidant to reduce the rate of formation of ROS under stress (See Scheme 1).



Scheme 1: Regulation of the pool size of *9-cis* βC. Red photon flux intensity controls the partitioning of electrons either for carotenoid biosynthesis or for photosynthesis, via energy absorption by chlorophyll and the PQ pool [1]. Red photon flux also controls phytochrome regulation of the production of gene transcripts for phytoene synthase and β-carotene isomerases. CHL A: chlorophyll a; P680: chlorophyll a, primary electron donor of Photosystem II; PQ_{ox}: plastoquinone, oxidised form; PQ_{red}: plastoquinone, reduced form; Cyt b_{6ox}: cytochrome b6f complex, oxidised form; $NADP^+$ NADP oxidised form; NADPH: NADP reduced form; PSY: phytoene synthase; 9-*cis*-βC-ISO: *9-cis* βC isomerase.

5. Conclusions

Red light availability regulates the isomerisation of *all-trans* β-carotene to *9-cis* β-carotene and upregulates carotenoid biosynthesis in the halotolerant microalga *Dunaliella salina.* In red light *9-cis* βC accumulated, caused by increase in the rate of isomerisation of *all-trans* βC to *9-cis* βC relative to the rate of its destruction. Red light may have industrial value as an energy-efficient light source for production of natural *9-cis* βC from *D. salina*.

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