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Uncovering the Potential of Nitrogen and Salt Stress for Enhanced β-Carotene Production and Antioxidant Capacity in Plant Pathogenic Alga *Cephaleuros*

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Abstract

A pure strain of *Cephaleuros* alga, designated *Cephaleuros* Cp.1, was successfully isolated directly from a citrus leaf lesion. This study investigated factors influencing both biomass and carotenoid accumulation in this green filamentous alga. Different nitrogen sources, NaCl stress, and trace elements in HSM, BBM, and Bristol media were compared. The autotrophic condition with HSM medium clearly offered the highest green biomass. Interestingly, *Cephaleuros* Cp.1 remained green in HSM using NH_4Cl as the nitrogen source but visibly changed to an orange hue due to the accumulation of β-carotene in BBM containing $NaNO_3$. This color change, along with the lower biomass and more intense yellow color when using nitrate, was the first reported in *Cephaleuros*, implying that nitrate may cause stress in the alga. Similar phenomena were clearly observed when NaCl was applied to HSM and BBM; on the other hand, Hutner's trace elements and trace metal solution had no significant effect. These findings suggest, for the first time, a link between stress conditions and the accumulation of β-carotene in *Cephaleuros* Cp.1. TLC revealed β-carotene as the main carotenoid accumulated by this alga. The accumulation was further enhanced by both nitrogen deficiency and salt stress. However, these stresses also led to a decrease in algal biomass. This study is the first to report free radical scavenging activity linked to β-carotene in *Cephaleuros*. Among the tested cultures, BBM exhibited the strongest activity (EC_{50} 1.40 mg/mL). These findings hold promise for future applications of *Cephaleuros* as a source of natural β-carotene with antioxidant properties.

Keywords: Algal Isolation, Carotenogenesis, Abiotic Stress, β-Carotene Antioxidant Activity

1. Introduction

Carotenoids are renowned for their potent antioxidant properties and as precursors to vitamin A. These lipid-soluble compounds are easily absorbed by the body. β-carotene, a prominent carotenoid, supports various tissues' health, including teeth, nails, hair, and vision tissue. Carotenoids exhibit strong antioxidant effects, guarding against oxidative reactions and offering potential anti-cancer and anti-inflammatory benefits, along with improved cardiovascular health. Researchers have turned

their focus to stimulating β-carotene synthesis and accumulation in green microalgae, such as *Dunaliella* spp., which can accumulate up to 14% of their dry weight in β-carotene. These green algae, classified within the Chlorophyta division, have been harnessed for the biosynthesis of carotenoids. Notable instances include the employment of *Dunaliella salina* to yield β-carotene, *Chlorella protothecoides* for lutein production, and *C. zofingiensis* and *Haematococcus pluvialis* for astaxanthin biosynthesis. Carotenoids within these algal cells function as accessory pigments, adept at absorbing light in spectral ranges unattainable by chlorophyll. Furthermore, these pigments act as a protective shield against the detrimental effects of excessive light or radiation.

Carotenoids found in green algae can be categorized into primary carotenoids, which are synthesized and stored within chloroplasts. In contrast, secondary carotenoids, such as canthaxanthin and astaxanthin, are produced when algae encounter unfavorable conditions or various stressors, including changes in temperature, nutrient availability, and light exposure. Secondary carotenoids are sequestered within lipid vesicles, where their primary role is to protect cells from potential damage, functioning as antioxidants (1, 2). Studies have revealed the ability of pathogenic algal genus *Cephaleuros* to synthesize secondary carotenoids, notably β-carotene and astaxanthin (3, 4). This ability is evident as many *Cephaleuros* species display a distinct orange hue, primarily due to the significant accumulation of these secondary carotenoids, visible to the naked eye.

Factors influencing the continuous production and accumulation of carotenoids in algae have gained interest, particularly the chemical factors. Algae, as photosynthetic organisms, closely link their ability to photosynthesize with their capacity to assimilate various nutrients and trace elements from their environment. Among these, nitrogen and phosphorus are crucial nutrients, and their availability in the environment directly impacts algal responses. Deprivation or imbalanced levels of these essential nutrients and improper salinity conditions can subject algae to environmental stressors, affecting their physiological responses (5, 6). In response to these pressures, algae may undergo structural modifications, particularly reducing the size of the chloroplast envelope to decrease starch production. Simultaneously, these conditions stimulate the accumulation of carotenoids within lipid vesicles, safeguarding cells from potential harm and damage by acting as antioxidants (7). Research findings have demonstrated that cultivating algae, such as *Dunaliella salina*, under limited nitrogen conditions can increase the accumulation of β-carotene (8). Similarly, astaxanthin accumulation in *Haematococcus pluvialis* has been enhanced under nitrogen-deficient culture conditions (9). Additionally, cultivating algae, like *D. salina*, in high salinity environments has led to the remarkable accumulation of β-carotene, reaching up to 14% of the dry weight (10).

Motivated by the potential for manipulating algal growth and metabolite production, this study investigates the cultivation of *Cephaleuros* Cp.1, a novel isolate from citrus leaf lesions infected with algal leaf spot disease. We compare the effects of three common culture media (HSM, BBM, and Bristol) on the algal biomass and the accumulation of specific compounds, particularly the carotenoid β-carotene. This research uniquely explores the influence of nitrogen source, salt stress, and trace element composition on pigment production. Additionally, we present the first ever assessment of the antioxidant properties of this β-carotene isolated from a plant pathogenic *Cephaleuros* alga.

2. Materials and Experiment

2.1 Isolation and cultivation of *Cephaleuros* alga

Citrus leaf fragments infected with *Cephaleuros* disease were selected as initial samples. These samples underwent a preliminary cleaning procedure, as described by (11), which involved rinsing them with tap water for one hour. Subsequently, the afflicted areas of the leaf fragments were cleaned with 70% ethanol-soaked cotton, followed by incisions using sterilized blades. The sections of the leaves that had undergone pathogen eradication were then introduced into a high salt medium (HSM) (12). Following this, the algae were rinsed before being transferred to fresh growth medium when visible growth was observed. This process of successive subculturing was repeated until pure axenic cultures of *Cephaleuros* were obtained. If necessary, antibiotics, such as streptomycin, were introduced to eliminate any residual

bacterial contaminants (13). The cultures were maintained at a constant temperature of 25 ± 2 °C under continuous illumination with a light intensity of 1300 lux.

2.2 Carotenoid profiling via TLC

The dried algal *Cephaleuros* Cp.1 sample weighing 10 mg was ground using a micro pestle in a 1.5 mL microcentrifuge tube until uniform. Sea sand (Nr.41 845, Ferak laboratory) was added to facilitate cell disruption. Subsequently, 400 µL of distilled water and 400 µL of methanol were added to the tube. The sample was vigorously mixed and kept in the dark at room temperature for 60 s. Then, 800 µL of chloroform was added, and the mixture was vigorously mixed again. The sample was then centrifuged at 8000 revolutions per minute for 5 s. The lower liquid phase was transferred to a new microcentrifuge tube and allowed to evaporate at room temperature in the dark. Subsequently, carotenoid compounds were analyzed through a thin-layer chromatography (TLC) procedure. The extracted compounds were dissolved in petroleum ether as per the method by (14). The compounds were spotted on a silica gel plate, which was then placed inside a tank containing a hexane and acetone mixture in a 3:1 ratio. After approximately 10 min, the plate was removed, and the migration distances of the *Cephaleuros* Cp.1 compounds, alongside a β-carotene standard (Sigma-Aldrich, Switzerland), were recorded for further analysis using the retention factor (R_f) values as specified in equation 2.1.

$$R_f = \frac{\text{Distance from the origin to the center of the compound spot}}{\text{Distance from the origin to the solvent front}} \quad (2.1)$$

2.3 Studying the impact of nitrogen, chloride, and trace elements on carotenoid accumulation

In order to assess the influence of different nitrogen sources, chloride (NaCl) presence, and trace element formulations on the accumulation of carotenoids in *Cephaleuros* Cp.1, two distinct growth media, HSM and Bold's basal medium (BBM), were employed, each with variations in nitrogen source (ammonium and nitrate) and chloride presence (with and without NaCl) as indicated in Table 1. Additionally, two trace element formulations, Hutner's trace elements and trace metals, were tested in combination with the aforementioned media and nutrient variations, resulting in a total of nine distinct experimental conditions. Approximately 10 mg fresh weight of *Cephaleuros* Cp. 1 was added as a starter culture into a 16x125 mm glass test tube containing 8 ml of liquid media mentioned earlier. This experiment was carried out at the same light intensity and temperature as stated in section 2.1. For a total of 60 days, changes in the algal filaments' colour were observed to analyze the influence of nutrient sources on carotenoid accumulation. After the 60-day cultivation period, algal biomass was photographed to document the changes in the thallus colour in the culture containers. The algal biomass was stored at -20 °C until further analysis.

Table 1 Media used in this study.

Main Component	HSM (mM)	BBM (mM)	Bristol (mM)
KH_2PO_4	5.44	1.29	1.29
K_2HPO_4	8.27	0.43	0.43
NH_4Cl	9.35	-	-
$NaNO_3$	-	2.94	2.94
$MgSO_4 \cdot 7H_2O$	0.08	0.30	0.30
$CaCl_2 \cdot 2H_2O$	0.07	0.17	0.17
NaCl	-	0.43	0.43
Trace metal solution	✓	✓	-

2.4 Analysis of antioxidant activity in algal extract using DPPH assay

After extracting the compounds from *Cephaleuros* Cp.1, which were dried and weighed at 15 mg, their antioxidant properties were assessed using a modified method based on (15). In this assay, 100 µL of the extract from *Cephaleuros* Cp.1 was mixed with 0.5 mL of 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution (Sigma-Aldrich, Germany). The mixture was then subjected to UV-visible spectrophotometry (Optizen 3220UV, Korea) at a wavelength of 515 nm. The initial absorbance (A_0) was recorded, and the mixture was left in darkness for 30 s. Afterward, the final absorbance (A_{30}) was measured. This process was conducted in triplicate. The percentage of DPPH inhibition was calculated using equation 2.2.

$$\%\ inhibition = \frac{A_0 - A_{30}}{A_0} \times 100 \quad (2.2)$$

The inhibition capability of the algal extract was quantified as Trolox equivalent antioxidant capacity (TEAC) and expressed in

µmol TE per gram of dry weight (µmol TE g^{-1} DW). Trolox (0.5 mM) dissolved in methanol was used as the standard for comparison. TEAC was determined by calculating the ratio of the slopes of the regression lines obtained for each sample and the Trolox standard.

3. Results and Discussion

3.1 Influence of algal media on the growth of *Cephaleuros* Cp.1

The research addresses the challenge posed by the relatively slow growth of the pathogenic *Cephaleuros* alga and explores the optimal nutrient conditions to stimulate the growth of *Cephaleuros* Cp.1 isolated from infected lime leaf (Figure 1).

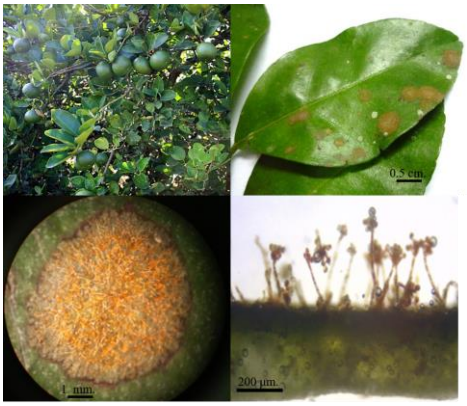

Figure 1 Lime fruits and leaves infected with *Cephaleuros*. The velvet colony of *Cephaleuros* Cp.1 (bottom left) resulted from its asexual reproductive structure (bottom right) containing orange pigment.

Over the 30 to 120-day period, intriguing variations in algal growth influenced by different nutrient media were observed (Table 2). HSM emerged as the most effective medium fostering a biomass increase from 5.8 ± 1.6 g/L on day 30 to a substantial 154.6 ± 15.6 g/L on day 120. This represented a remarkable 26.6-fold increase in biomass. BBM displayed commendable growth, progressing from 5.4 ± 1.9 g/L on day 30 to 26.1 ± 1.2 g/L on day 120, marking a 4.8-fold increase in biomass.

Table 2 The effects of nutrient types on the biomass of *Cephaleuros* Cp.1.

Duration of cultivation (Days)/ Culture media	Biomass (mg)
30 days	
HSM	5.8 ± 1.6^{b}
BBM	5.4 ± 1.9^{b}
Bristol	2.4 ± 0.6^{a}
60 days	
HSM	53.2 ± 4.1^{c}
BBM	14.2 ± 2.6^{b}
Bristol	9.3 ± 1.6^{a}
90 days	
HSM	81.4 ± 16.6^{b}
BBM	21.4 ± 1.5^{a}
Bristol	17.6 ± 3.0^{a}
120 days	
HSM	154.6 ± 15.6^{b}
BBM	26.1 ± 1.2^{a}
Bristol	22.2 ± 5.1^{a}

Note: Superscript letters indicate statistically significant differences ($p < 0.05$).

In contrast, Bristol medium showed less favorable growth patterns, maintaining lower biomass levels at 2.4 ± 0.6 g/L on day 30 and reaching 22.2 ± 5.1 g/L on day 120. These findings underscore the pivotal role of nutrient media in shaping *Cephaleuros* Cp.1 growth, with HSM providing an exceptionally conducive environment for substantial biomass enhancement. The results indicate that HSM was the most effective medium for stimulating algal growth, with biomass increasing nearly 27-fold from day 30 to day 120. This could be the result of more enriched nutrients, especially nitrogen and phosphorus, that are present in HSM (Table 1). In addition, *Cephaleuros* Cp.1 could prefer ammonium over nitrate as a nitrogen source due to its lower energy requirement for assimilation (16,17). While BBM also exhibited significant growth, it couldn't match the exceptional biomass increase seen in HSM. Although Bristol was less favorable, it still managed to support notable growth over the 120-day period.

Additionally, an intriguing phenomenon was observed when *Cephaleuros* Cp.1 was cultivated in both BBM and Bristol media. By the end of the second month of cultivation, the algal cultures underwent a striking transformation in colouration, transitioning from green to yellow.

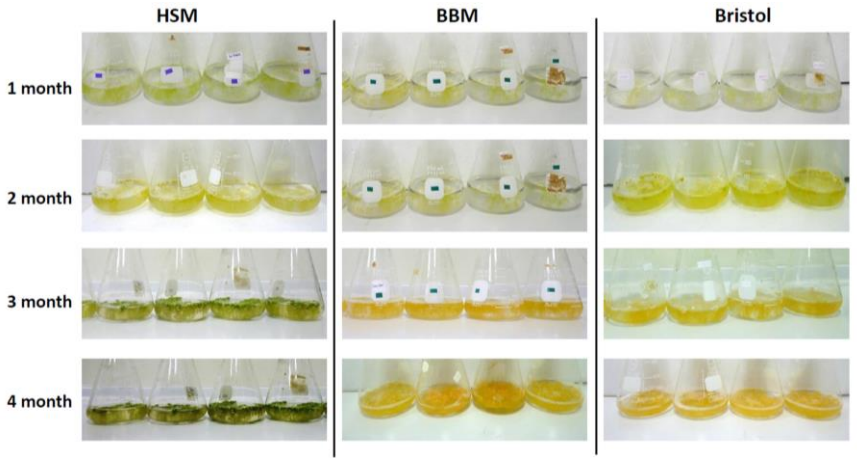

Figure 2 Growth and colour shift of Cephaleuros Cp.1 in three different nutrient media

Subsequently, the algae gradually shifted to a distinct orange hue during the third month (Figure 2). These shifts in pigmentation open avenues for further exploration into the underlying physiological and biochemical processes of *Cephaleuros* Cp.1 in response to varying nutrient conditions.

3.2 Pigments in *Cephaleuros* Cp.1

Based on the observations of colour shifts in *Cephaleuros* alga when cultivated in three different nutrient media, the analysis of pigment types in 120-day-old algal biomass cultured in HSM and BBM revealed distinct results. The extraction of dried algae samples using methanol following Grung et al.'s method (14) and subsequent analysis by TLC indicated a clear difference in pigment composition. *Cephaleuros* Cp.1 grown in HSM contained five distinct pigments: lutein, chlorophyll a, chlorophyll b, pheophytin, and β-carotene. In contrast, algae cultivated in BBM primarily exhibited β-carotene as the only dominant pigment. These findings suggest that the nutrient media significantly impact the accumulation of pigments, particularly β-carotene, as evidenced by the absence of other pigment types in BBM-grown algae compared to those in HSM (Figure 3, lane 2 and lane 3).

In algal cultivation, the choice of a nitrogen source is critical, with ammonium (NH_4^+), nitrate (NO_3), and urea being common options. Ammonium is generally preferred due to its efficient uptake compared to other nitrogen sources (16-18). This aligns with studies by Enwereuzoh and Onyeagoro, 2014 (8) and Giordano, 2001 (19), where ammonium-rich media led to faster growth and higher biomass yield in green algae, such as *Dunaliella salina*. Our recent experiment confirmed that *Cephaleuros* Cp.1's biomass production and growth benefited significantly from the high ammonium nitrogen content in the HSM medium, surpassing BBM and Bristol media. Ammonium's effectiveness in various biological processes, promoting rapid growth and biomass accumulation, plays a key role.

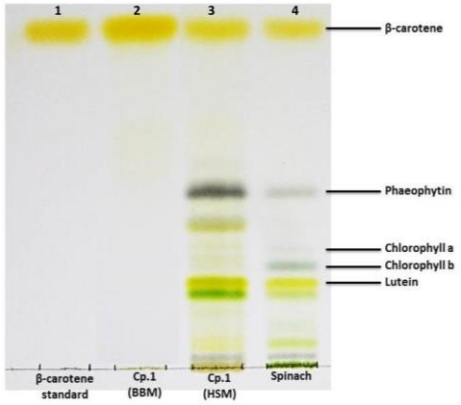

Figure 3 Pigment profiles of *Cephaleuros* Cp.1 cultivated in BBM and HSM nutrient media. Lane 1: β-carotene standard, Lane 2: *Cephaleuros* Cp.1 extract cultivated in BBM, Lane 3 *Cephaleuros* Cp.1 extract cultivated in and HSM, and Lane 4: Spinach leaf extract.

3.3 Impact of nitrogen source, NaCl, and trace elements on the accumulation of pigments in *Cephaleuros* Cp.1

In the investigation of factors influencing the physiology and pigmentation of *Cephaleuros* Cp.1, the role of nitrogen source, sodium chloride, and trace elements were explored. The following experiments shed light on how each of these elements impact the colouration and pigment accumulation in this unique algae species. In HSM, replacing the nitrogen source from NH_4Cl to $NaNO_3$ resulted in a colour shift of *Cephaleuros* Cp.1 from green (Figure 4: 1A) to yellow (Figure 4: 1B). Conversely, in BBM, changing the nitrogen source from $NaNO_3$ to NH_4Cl led to the algae changing from yellow (Figure 4: 1D) to green (Figure 4: 1C). These experiments demonstrate the significant impact of nitrogen source on the accumulation of β-carotene in *Cephaleuros* Cp.1. Specifically, $NaNO_3$ in the nutrient medium stimulated increased β-carotene accumulation within the algal filaments. This is further supported by the observed colour change when switching from $NaNO_3$ to NH_4Cl in BBM. The algae initially cultured in BBM with NH_4Cl shifted from yellow to green, indicating both a change in pigmentation and enhanced growth.

In the case of HSM, which typically does not contain added sodium chloride (NaCl), the colour of the *Cephaleuros* Cp.1 remained green (Figure 4: 2A). However, when sodium chloride was introduced into the medium (at the same concentration as in BBM) for 60 days, there was a slight colour change observed in some parts of the algae, transitioning from green to a pale green-yellow hue (Figure 4: 2B). Nonetheless, this colour shift was not as distinct as the colour changes seen in algae cultivated in BBM media. Likewise, when NaCl supplementation was omitted from BBM medium, there was no significant impact on the algae's colour, which continued to exhibit its normal orange shade (Figure 4: 2C-D). These experimental results indicate that the addition of sodium chloride to HSM does not induce significant colour changes in *Cephaleuros*. Similarly, the presence or absence of salt in BBM has minimal effect on the algae's colouration. In both HSM and BBM, the addition of Hutner's trace element solution and trace metal solution yielded consistent colouration in *Cephaleuros* Cp.1.

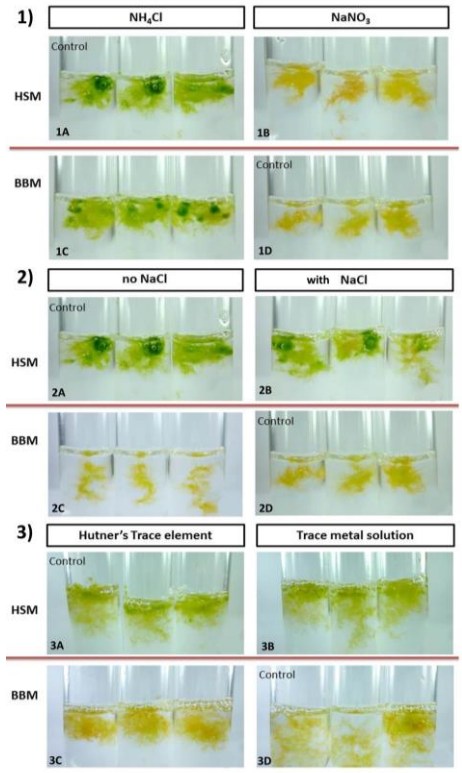

Figure 4 Effect of nitrogen source (1), NaCl (2) and trace elements (3) on colour shift of *Cephaleuros* Cp.1 at 60 days culture. 1A: HSM (control), 1B: HSM/$NaNO_3$, 1C: BBM/NH_4Cl, 1D: BBM (control), 2A: HSM (control), 2B: HSM/NaCl, 2C: BBM/no NaCl, 2D: BBM (control), 3A: HSM (control), 3B: HSM/ Trace metal solution, 3C: BBM/ Hutner's trace element, and 3D: BBM (control).

The algae appeared yellow/green in the presence of these trace element solutions in both nutrient media (Figure 4: 3A-D).

High salinity levels and the composition of trace elements in nutrient formulations also influence algal biomass production. Salinity can hinder growth and metabolic processes in various algal species (20,21). The trace element composition, including solutions like Hutner's trace metal solution, affects mineral content and growth responses (22). Optimizing salt levels and trace element composition in cultivation strategies is crucial for specific algal species and growth conditions (23).

The type and quantity of nitrogen sources directly impact carotenoid accumulation in algae. Nitrate as a nitrogen source can promote the accumulation of compounds like β-carotene in some algal species (24,25). Ammonium is generally a more favorable nitrogen form for algae, while nitrate and urea can induce stress and enhance pigment accumulation (17,18,26). This variability in nitrogen sources affects biomass and pigment accumulation in *Cephaleuros* Cp.1. Similar to other algae, nitrogen starvation can induce oxidative stress in *Cephaleuros* Cp.1, leading to enhanced carotenoid production (27–30). Including chloride salt in cultivation can enhance carotenoid levels, but our experiment used relatively low concentrations (31–33). Higher chloride concentrations may lead to more pronounced carotenoid accumulation and colour shifts, which were not observed in our study with *Cephaleuros* Cp.1

The importance of trace elements, including cobalt (Co_2^+), iron (Fe_3^+), molybdenum (Mo_2^+), and manganese (Mn_2^+), for algae growth is well-established (34). However, specific effects can vary based on growth conditions and algal species (34). Additional research is needed to clarify these particular effects.

3.4 Antioxidant activity of *Cephaleuros* Cp.1 extract

When analyzing and comparing the antioxidant activity of *Cephaleuros* Cp.1 extracts (dry weight 15 mg) cultured in HSM, BBM, and Bristol nutrient media for 60 days, it was observed that the % inhibition correlated with the concentration of the algal extracts. The antioxidant activity was determined based on the Trolox standard curve (R^2 = 0.9876) and calculating the EC_{50} values (Table 3) from the respective graphs (data not shown). The algal extract from HSM had an EC_{50} value of 2.39 ± 0.07 mg/mL. In contrast, the algal extracts from BBM and Bristol had EC_{50} values of 1.44 ± 0.02 mg/mL and 1.46 ± 0.15 mg/mL, respectively. These results suggest that *Cephaleuros* Cp.1 cultured in BBM and Bristol nutrient media exhibited similar EC_{50} values. Importantly, the EC_{50} value for the algal extract from HSM was notably higher, at 2.39 mg/mL. This indicates that *Cephaleuros* alga cultured in BBM and Bristol media have a higher antioxidant capacity compared to those cultured in HSM, up to 1.5 times greater. On the other hand, the algal extracts from BBM and Bristol showed carotenoid accumulation up to 9 times higher than that of the algal extract from HSM.

In the context of antioxidants, brown seaweed has been a primary focus due to its rich xanthophyll carotenoids (35,36). Our study found that *Cephaleuros* exhibited higher antioxidant properties, although variations in extraction methods, durations, solvents, and techniques can influence compound concentrations (37). A uniform extraction method and concurrent testing are recommended for accurate antioxidant property comparisons.

Table 3 EC_{50} of algal extracts grown in three media.

Algal extract	EC_{50} (mg)
HSM	2.12 ± 0.14^a
BBM	1.47 ± 0.05^b
Bristol	1.45 ± 0.05^b

4. Conclusions

Our study provides the first experimental evidence that stress conditions, induced by nitrogen starvation and chloride salt, promote β-carotene accumulation in a plant pathogenic *Cephaleuros* alga. Prior research lacked investigation into these factors for this specific green alga, potentially due to limited cultivation efforts. Here, we demonstrate that an autotrophic condition with HSM medium is most favorable for *Cephaleuros* Cp.1 biomass production (approximately 7 times higher). Interestingly, the alga responded to stress conditions by significantly accumulating the valuable β-carotene, a pigment not previously reported in *Cephaleuros*. Furthermore, this study is the first to report the natural antioxidant activity associated with this β-carotene in *Cephaleuros*. These findings unveil the potential of utilizing *Cephaleuros*, the causative agent of algal leaf spot disease, as a novel source for both natural β-carotene and its intrinsic antioxidant properties. Future research focused on optimizing cultivation strategies and enhancing these functionalities could position *Cephaleuros* as a valuable resource for future biotechnological applications.

Declaration of Conflicting Interests

The authors declared that they have no conflicts of interest in the research, authorship, and this article's publication.

References

1. Huang JJ, Lin S, Xu W, Cheung PCK. Occurrence and biosynthesis of carotenoids in phytoplankton. Biotechnol Adv. 2017 Sep 1;35(5):597–618.
2. Takaichi S. Carotenoids in algae: distributions, biosyntheses and functions. Mar Drugs. 2011 Jun;9(6):1101–18.
3. Lopez-Bautista JM. The Trentepohliales revisited. Louisiana State University; 2002.
4. Thompson RH, Wujek DE. Trentepohliales: *Cephaleuros, Phycopeltis, and Stomatochroon.* Morphology, taxonomy, and ecology. Enfield, New Hampshire (US): Science Publishers; 1997.
5. Barone ME, Parkes R, Herbert H, McDonnell A, Conlon T, Aranyos A, et al. Comparative response of marine microalgae to H_2O_2-induced oxidative Stress. Appl Biochem Biotechnol. 2021;193(12):4052–67.
6. Parkes R, Barone ME, Herbert H, Gillespie E, Touzet N. Antioxidant activity and carotenoid content responses of three *Haematococcus* sp. (Chlorophyta) strains Exposed to Multiple Stressors. Appl Biochem Biotechnol. 2022;194(10):4492–510.
7. Shi TQ, Wang LR, Zhang ZX, Sun XM, Huang H. Stresses as first-line tools for enhancing lipid and carotenoid production in microalgae. Front Bioeng Biotechnol. 2020;8:610.
8. Enwereuzoh UO, Onyeagoro GN. A novel aeration method for the preparation of algae (*Dunaliella Salina*) biomass for biofuel production. Am J Eng Res. 2014;3(9):209–14.
9. Christian D, Zhang J, Sawdon AJ, Peng CA. Enhanced astaxanthin accumulation in *Haematococcus pluvialis* using high carbon dioxide concentration and light illumination. Bioresour Technol. 2018;256:548–51.
10. Wolf L, Cummings T, Müller K, Reppke M, Volkmar M, Weuster-Botz D. Production of β-carotene with *Dunaliella salina* CCAP19/18 at physically simulated outdoor conditions. Eng Life Sci. 2021; 21(3–4):115–25.
11. Suto Y, Ohtani S. Morphology and taxonomy of five *Cephaleuros* species (Trentepohliaceae, Chlorophyta) from Japan, including three new species. Phycologia. 2009;48(4):213–36.
12. Sueoka N, Chiang KS, Kates JR. Deoxyribonucleic acid replication in meiosis of *Chlamydomonas reinhardi*: I. isotopic transfer experiments with a strain producing eight zoospores. J Mol Biol. 1967;25(1):47–66.
13. Suto Y, Ohtani S. Strigula smaragdula complex (Lichenized Ascomycota, Strigulaceae) on living leaves of woody plants from Shimane-ken, Western Japan. Lichenology. 2011;10(1):1–13.
14. Grung M, D'Souza FML, Borowitzka M, Liaaen-Jensen S. Algal carotenoids 51. Secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (3S, 3′S)-astaxanthin esters. J Appl Phycol. 1992;4(2):165–71.
15. Saini RK, Shetty NP, Prakash M, Giridhar P. Effect of dehydration methods on retention of carotenoids, tocopherols, ascorbic acid and antioxidant activity in *Moringa oleifera* leaves and preparation of a RTE product. J Food Sci Technol. 2014; 51(9):2176–82.
16. Hellebust JA, Ahmad I. Regulation of nitrogen assimilation in green microalgae. Biol Oceanogr. 1989;6(3–4):241–55.
17. Kumar A, Bera S. Revisiting nitrogen utilization in algae: A review on the process of regulation and assimilation. Bioresour Technol Rep. 2020;12:100584.
18. Silva NFP, Gonçalves AL, Moreira FC, Silva TFCV, Martins FG, Alvim-Ferraz MCM, et al. Towards sustainable microalgal biomass production by phycoremediation of a synthetic wastewater: A kinetic study. Algal Res. 2015;11:350–8.
19. Giordano M. Interactions between C and N metabolism in *Dunaliella salina* cells cultured at elevated CO_2 and high N concentrations. J Plant Physiol. 2001; 158(5):577–81.
20. Almahrouqi H, Naqqiuddin MA, Achankunju J, Omar H, Ismail A. Different salinity effects on the mass cultivation of Spirulina (*Arthrospira platensis*) under sheltered outdoor conditions in Oman and Malaysia. J Algal Biomass Util. 2015;6:1–14.

21. Moronta R, Mora R, Morales E, Moronta R, Mora R, Morales E. Response of the microalga *Chlorella sorokiniana* to pH, salinity and temperature in axenic and non axenic conditions. Rev Fac Agron Univ Zulia. 2006;23(1):28–43.
22. Ambati RR, Gogisetty D, Aswathanarayana RG, Ravi S, Bikkina PN, Bo L, et al. Industrial potential of carotenoid pigments from microalgae: Current trends and future prospects. Crit Rev Food Sci Nutr. 2019; 59(12):1880–902.
23. Panahi Y, Yari Khosroushahi A, Sahebkar A, Heidari HR. Impact of cultivation condition and media content on chlorella vulgaris composition. Adv Pharm Bull. 2019;9(2):182–94.
24. Li X, Li W, Zhai J, Wei H. Effect of nitrogen limitation on biochemical composition and photosynthetic performance for fed-batch mixotrophic cultivation of microalga *Spirulina platensis*. Bioresour Technol. 2018;263: 555–61.
25. Li X, Li W, Zhai J, Wei H, Wang Q. Effect of ammonium nitrogen on microalgal growth, biochemical composition and photosynthetic performance in mixotrophic cultivation. Bioresour Technol. 2019;273: 368–76.
26. Chowdury KH, Nahar N, Deb UK. The growth factors involved in microalgae cultivation for biofuel production: a review. Comput Water Energy Environ Eng. 2020;9(4):185–215.
27. Zhang YM, Chen H, He CL, Wang Q. Nitrogen starvation induced oxidative stress in an oil-producing green alga *Chlorella sorokiniana* C3. PLOS ONE. 2013;8(7):e69225.
28. Wu M, Zhu R, Lu J, Lei A, Zhu H, Hu Z, et al. Effects of different abiotic stresses on carotenoid and fatty acid metabolism in the green microalga *Dunaliella salina* Y6. Ann Microbiol. 2020;70(1):48.
29. Mulders KJM, Janssen JH, Martens DE, Wijffels RH, Lamers PP. Effect of biomass concentration on secondary carotenoids and triacylglycerol (TAG) accumulation in nitrogen-depleted *Chlorella zofingiensis*. Algal Res. 2014;6(Part A):8–16.
30. Urreta I, Ikaran Z, Janices I, Ibañez E, Castro-Puyana M, Castañón S, et al. Revalorization of *Neochloris oleoabundans* biomass as source of biodiesel by concurrent production of lipids and carotenoids. Algal Res. 2014;5:16–22.
31. Del Campo JA, García-González M, Guerrero MG. Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. Appl Microbiol Biotechnol. 2007;74(6):1163–74.
32. Pelah D, Sintov A, Cohen E. The effect of salt stress on the production of canthaxanthin and astaxanthin by *Chlorella zofingiensis* grown under limited light intensity. World J Microbiol Biotechnol. 2004;20(5):483–6.
33. Sun XM, Ren LJ, Zhao QY, Ji XJ, Huang H. Microalgae for the production of lipid and carotenoids: a review with focus on stress regulation and adaptation. Biotechnol Biofuels. 2018;11(1):272.
34. Chen M, Tang H, Ma H, Holland TC, Ng KYS, Salley SO. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresour Technol. 2011;102(2):1649–55.
35. Begum R, Howlader S, Mamun-Or-Rashid ANM, Rafiquzzaman SM, Ashraf GM, Albadrani GM, et al. Antioxidant and signal-modulating effects of brown seaweed-derived compounds against oxidative stress-associated pathology. Oxid Med Cell Longev. 2021;2021:e9974890.
36. Pereira AG, Otero P, Echave J, Carreira-Casais A, Chamorro F, Collazo N, et al. Xanthophylls from the Sea: algae as source of bioactive carotenoids. Mar Drugs. 2021; 19(4):188.
37. Kokabi M, Yousefzadi, Yousefzadi M, Ahmadi A, Feghhi, Amin M, et al. Antioxidant activity of extracts of selected algae from the Persian Gulf, Iran. J Persian Gulf. 2013;4:45–50.

