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Effects of exogenous hormones on the regeneration of juveniles from *Sargassum fusiforme* holdfasts

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During the cultivation of *Sargassum fusiforme*, sexual reproduction of superior strains can hinder the stable inheritance of their desirable traits and reduce their economic benefits. However, vegetative reproduction of *S. fusiforme* holdfasts has high potential for subculture. In this study, we investigated the effects of different concentrations of α-naphthaleneacetic acid (NAA), gibberellin (GA_3), and 6-benzyladenine (BAP) on the growth and regeneration of *S. fusiforme* holdfasts. Exogenous application of 1.5, 2 $mg{\cdot}L^{-1}$NAA, 1.5 $mg{\cdot}L^{-1}$ GA_3 or 2, 3 $mg{\cdot}L^{-1}$BAP significantly promoted photosynthetic activity and the absorption and utilization of nitrate nitrogen in *S. fusiforme* holdfasts, thus improving the relative growth rate (RGR) and regeneration rate (RR) and shortening the time for the regeneration of juveniles from *S. fusiforme* holdfasts to around the 6th day. Among these, the RGR and RR at the end of the culture period with 1.5 $mg{\cdot}L^{-1}$ of NAA increased by 118.9% and 67.4%, respectively, compared with those of the control group. However, treatment with 1.5 $mg{\cdot}L^{-1}$ GA_3 increased the RR of *S. fusiforme* holdfasts by 58.8% compared with that of the control group. Under BAP treatment at 3 $mg{\cdot}L^{-1}$, the RR of *S. fusiforme* holdfasts increased by 23.4% compared with that of the control group; its promoting effect was thus weaker than that of NAA and GA_3. When the concentration of GA_3 or BAP was too high (3 $mg{\cdot}L^{-1}$; 5 $mg{\cdot}L^{-1}$), the RR of the holdfasts decreased by 46.5% and 42.8%, respectively compared with that of the control group. Therefore, exogenous application of NAA at 1.5 $mg{\cdot}L^{-1}$, GA_3 at 1.5 $mg{\cdot}L^{-1}$, and BAP at 3 $mg{\cdot}L^{-1}$ can be used to induce regeneration of *S. fusiforme* holdfasts, shorten the culture time of regenerated seedlings, and obtain more regenerative seedlings, thereby improving economic efficiency.

KEYWORDS

***Sargassum fusiforme*, holdfast regeneration, seaweed hormones, breeding, plant growth regulators**

1 Introduction

Sargassum fusiforme (Harvey) Okamura (Sargassaceae, Phaeophyta) is a perennial macroalga endemic to the nearshore waters of the northwestern Pacific Ocean. Studies have shown that fucoidan, an extract of *S. fusiforme*, can improve metabolic disorders related to obesity and reduce visceral fat accumulation; further, it is rich in amino acids and trace elements, which have high nutritional and medicinal value (Liu et al., 2021; Zuo et al., 2022). *S. fusiforme* is a dioecious plant that can reproduce sexually or asexually. Sexual reproduction occurs through the release and union of male and female gametes from male and female plants, respectively with the conspecifics then growing and developing into *S. fusiforme* juveniles (Lin et al., 2021). In contrast to sexual reproduction, asexual reproduction generally occurs during the late stage of growth. When the seawater temperature rises, the leaves gradually fall off, leaving only the holdfast; when the seawater temperature drops, the holdfast regenerates juveniles (Yoshida and Shimabukuro, 2017; Xu et al., 2022a). Moreover, it has been reported that several *Sargassum* species, including *Sargassum fusiforme*, regenerate juveniles through holdfast. These studies also showed that *S. fusiforme* holdfast had higher tolerance to high temperature and low nutrient conditions during summer (Ito et al., 2009; Yatsuya et al., 2012; Loffler et al., 2018; Endo et al., 2021). Although superior traits of juveniles can be stably maintained through asexual propagation *via* holdfasts, the number of seedlings produced by holdfasts is currently limited. Therefore, a method to increase the regeneration rate of holdfasts is urgently needed in *S. fusiforme* culture and is important for increasing the source of *S. fusiforme* seedlings, especially for stable multiplication of the germplasm of screened desirable cultured strains.

Phytohormones are organic signaling molecules produced by plants through their metabolism and can produce significant physiological effects at low concentrations. The main widely reported plant hormones include auxins, cytokinins, gibberellin, abscisic acid, ethylene (Xiong et al., 2009; Iqbal et al., 2014; Miyakawa and Tanokura, 2017; Waadt, 2020). Numerous studies have shown that although seaweeds have a simple morphology, they share many similarities with vascular plants. Phytohormones control most of the growth and developmental processes in vascular plants; they are also present in seaweeds and may have similar functions in seaweeds (Stirk and Van Staden, 2014). Plant growth regulators (PGRs), are organic compounds that are synthetic (or naturally derived from microorganisms) and have growth and developmental regulatory effects similar to those of natural plant hormones (Rademacher, 2015). Plant hormones such as auxin, gibberellin, and cytokinin can be exogenously applied as PGRs to regulate the growth and reproduction of plants and seaweeds (Duran-Medina et al., 2017; Miyakawa and Tanokura, 2017; Song et al., 2020; Hernandez-Garcia et al., 2021; Nedved et al., 2021; Jiksing et al., 2022). Chen et al. (2022) reported that exogenous application of auxin can promote biomass production and branching in *Gracilariopsis lemaneiformis*. Further, exogenous addition of PGRs can direct regeneration and callus induction in *Sargassum polycystum* C. Agardh (Muhamad et al., 2018). Exogenous addition of PGRs may also have a corresponding effect during the germination of *S. fusiforme* holdfasts.

α-Naphthaleneacetic acid (NAA), gibberellin (GA_3), and 6-benzyladenine (BAP) have been reported to promote the growth of macro-green seaweed (Wei et al., 2020). However, the optimal concentrations of the above mentioned PGRs to promote the regeneration of *S. fusiforme* holdfasts remain unclear. Therefore, it is crucial to determine the optimal concentrations of NAA, BAP, and GA_3 for promoting the regeneration of *S. fusiforme* holdfasts. In this study, we exogenously administered PGRs to investigate their effects on the physiological properties of *S. fusiforme* holdfasts and the regenerated juveniles. This study has practical significance for the breeding of holdfast seedlings of the economic seaweed *S. fusiforme* and for the establishment of a stable genetic culture method for superior strains.

2 Materials and methods

2.1 Sample collection and experimental design

In this study, the residual holdfasts from the mature harvest of *S. fusiforme* culture rafts in Dongtou District, Wenzhou City, Zhejiang Province, were taken and brought back to the laboratory at a low temperature. All *S. fusiforme* holdfasts were collected from the cultured rafts for superior strains. After washing the epiphytes and impurities on the holdfast surface using seawater, it was soaked for 2 min in 0.38% NaClO and 0.5% KI (Kerrison et al., 2015; Xu et al., 2022a). The soaked holdfast was washed thrice with sterilized seawater, followed by adaptive cultivation for 3 h. Holdfasts under similar conditions were selected for cultivation in groups. Except for the control group, the exogenously applied hormone concentrations during the culture were as follows: α-naphthaleneacetic acid (NAA): 0.5, 1, 1.5, 2.0, and 3.0 $mg{\cdot}L^{-1}$; gibberellin (GA_3): 0.5, 1, 1.5, 2.0, and 3.0 $mg{\cdot}L^{-1}$; and 6-benzyladenine (BAP): 1, 2, 3, and 5 $mg{\cdot}L^{-1}$ (Wei et al., 2020). Each group had three replicates, and each replicated had three *S. fusiforme* holdfasts.

2.2 Determinations of the relative growth rate and regeneration rate

The medium (29‰ seawater, 200 $\mu mol{\cdot}L^{-1}$ $NaNO_3$, 20 $\mu mol{\cdot}L^{-1}$ NaH_2PO_4) was changed every three days (Zhang et al., 2020); the holdfast was weighed, photographed, and recorded for regeneration. The relative growth rate (RGR) and

regeneration rate (RR) were calculated as follows:

$$RGR\left(\%\cdot d^{-1}\right) = [\ln(W_t/W_0)/t] \times 100$$

$$RR(\%) = \left(\frac{\text{Number of swellings regenerated from holdfasts}}{\text{Number of holdfasts}}\right) \times 100$$

where W_0 is the initial weight of the holdfast, and W_t is the weight after t days of incubation.

2.3 Determination of photosynthetic activity

Chlorophyll fluorescence kinetics in *S. fusiforme* holdfasts were determined using Junior-Pam (Walz, Effeltrich, Germany) (Hong et al., 2021). After dark adaptation of the samples for 30 min, the electron transfer rate (rETR) was measured at eight preset incremental light intensities in the range of 0–820 $\mu mol\cdot m^{-2}\cdot s^{-1}$ in the area near the tip of the filamentous holdfasts of *S. fusiforme*, and a fast light curve (RLC) was fitted according to Platt's formula (Platt et al., 1980):

$$rETR\left(\mu mol\ electrons\cdot m^{-2}\cdot s^{-1}\right) = rETRm\cdot\left(1-e^{-\alpha\cdot PAR/rETRm}\right)\cdot e^{-\beta\cdot PAR/rETRm}$$

where rETR is the relative electron transfer rate, rETRm is the maximum relative electron transfer rate, α is the initial slope, reflecting the efficiency of light energy utilization,and β is the photoinhibition parameter.

2.4 Determinations of the maximum net photosynthetic rate and dark respiration rate

After weighing and recording, the holdfast was added to 200 mL of incubated seawater and rotated at a constant speed using a magnetic stirrer at a constant temperature in a water bath (20° C). The light intensity was adjusted to saturation light intensity (400 $\mu mol\cdot m^{-2}\cdot s^{-1}$) using LED light, the samples were light-adapted for 3–5 min, and the oxygen content was measured every 30 s using an optical fiber dissolved oxygen meter (Pyro Science FireSting O_2, Germany). The above steps were repeated, and maximum net photosynthetic rate (Pm) was obtained after calculation.

Next, the LED lamp and magnetic stirrer were switched off, the *S. fusiforme* holdfasts were placed in a light-proof state, and acclimatized to dark conditions for 3–5 min. The magnetic stirrer was turned on; after the reading was stabilized, the readings were recorded using the same method described above, and the dark respiration rate of holdfasts (Rd) were calculated. Both the Pm and Rd were calculated in $\mu mol\ O_2\cdot g^{-1}Fw\cdot h^{-1}$. The dissolved oxygen standard was calculated as per the formula shown below (Henley, 1993):

$$P\ (\mu mol\ O_2\cdot g^{-1}Fw\cdot h^{-1}) = (\Delta\cdot\alpha\cdot V\cdot 0.0375)/Fw$$

Where Δ is the average value of the difference between the measured values, α is the satiation dissolved oxygen constant at each temperature, and V is the volume of the reaction fluid (mL).

2.5 Determination of the nitrate-nitrogen uptake rate

The nitrate-nitrogen content in the culture medium before absorption and after 24 h was determined in seawater samples collected at the respective time points using the zinc-cadmium reduction method (Sun et al., 2013). The rate of nitrate-nitrogen uptake by *S. fusiforme* holdfasts in the culture solution was expressed as the rate of reduction of nitrate-nitrogen per hour ($\mu mol\ NO^{3-}\cdot g^{-1}\cdot h^{-1}$). The calculation formula used is as follows:

$$\text{Nitrate} - \text{N uptake rate}(\mu mol\ \ NO^{3-}\cdot g^{-1}\cdot h^{1}) = (N_0 - N_t)\times V/M/t$$

where N_0 is the initial nitrogen concentration of culture solution, N_t is the final nitrogen concentration of culture solution at the time of determination, V is the volume of culture solution (L), M is the wet weight of *S. fusiforme* holdfasts (g), and t is the incubation time (h).

2.6 Determination of nitrate reductase activity

Nitrate reductase (NR) activity was measured according to Corzo's method (Corzo and Niell, 1991). Briefly, 0.1 g of *S. fusiforme* holdfasts was mixed with 10 mL of reaction medium [0.1 M NaH_2PO_4(buffer, pH 7.5), 0.5 mM EDTA, 0.1% (v/v) propanol, 0.01 mM glucose, 50 mM $NaNO_3$] and aerated with N_2 for 10 min, the reaction system was then scaled and incubated for 30 min at 30°C in a dark environment. The supernatant reaction solution (4 mL) was then mixed with 2 mL of 1% sulfonamide and 2 mL of 0.1% N-(1-naphthyl)-ethylenediamine hydrochloride in a test tube and allowed to for 15 min. Absorbance was then measured at 543 nm. The NR activity of *S. fusiforme* holdfasts was expressed as $\mu mol\ NO_2^{-1}\cdot g^{-1}Fw\cdot h^{-1}$.

2.7 Determination of glutamine synthetase activity

For determining the glutamine synthetase (GS) activity 0.1 g of *S. fusiforme* holdfasts was mixed with 5 mL of extraction buffer (pH 8.0) containing 50 $mmol\cdot L^{-1}$ Tris-HCl, 1 $mmol\cdot L^{-1}$ EDTA, 10 $mmol\cdot L^{-1}$ β-mercaptoethanol, 5 $mmol\cdot L^{-1}$

dithiothreitol (DTT), 10.0 mmol·L^{-1} $MgCl_2$·7 H_2O, 6.6% of polyvinylpolypyrrolidone (PVPP), 0.5 mmol·L^{-1} phenylmethylsulfonyl (PMSF), and 1 mmol·L^{-1} cysteine. This was followed by centrifugation at 17000 × *g* for 20 min at 4°C. The supernatant was collected and the absorbance value was measured at 540 nm. γ-Glutamyl isohydroxamic acid was used to generate the standard curve. One unit of GS activity was defined as the amount of enzyme required to catalyze the production of 1 μmol of γ-glutamyl isohydroxamic acid per minute at 25°C (Setien et al., 2013).

2.8 Determination of glutamate synthase activity

Approximately 0.1 g of *S. fusiforme* holdfasts was weighed and finely ground with a small amount of quartz sand and 6 mL of enzyme extract [10 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM mercaptoethanol]. The mixture was centrifuged for 30 min (12000 × *g*, 4°C), 3 mL of the supernatant enzyme reaction solution was collected, and the change in absorbance was measured at 340 nm. One unit of glutamate synthase (GS) activity was defined as consumption of 1 nmol NADH in 1 min by 1 g of tissueand was expressed as nmol·min^{-1}·g^{-1} (Shah et al., 2017).

2.9 Determination of soluble protein content

Soluble proteins in holdfasts were determined using the Coomassie Brilliant Blue G-250 dye binding method (Kochert, 1978). Holdfasts (0.1 g) were added to 5 mL of phosphate buffer (pH 6.8) and ground at low temperature. After centrifugation at 4°C and 4000 rpm for 10 min, 1 mL of supernatant was collected, mixed with 4 mL of Coomassie Brilliant Blue G-250 staining solution, and left for 3 min. The absorbance of the samples was measured at 595 nm. A standard curve (bovine serum albumin standard solution) was used to calculate the soluble protein (SP) content (mg·g^{-1}Fw) of *S. fusiforme* holdfasts.

2.10 Determination of soluble carbohydrates

Glucose was used to generate the standard curve, and the anthrone method was used for soluble carbohydrates determination (Ershadi et al., 2015). Briefly, holdfasts (0.1 g) were ground thoroughly, mixed well with 9 mL of distilled water and 1 mL of magnesium carbonate suspension, and centrifuged at 4°C for 10 min at 10000 rpm. The supernatant (1 mL) was mixed well with 2 mL of distilled water and 10 mL of anthrone reagent, placed in a boiling water bath at 100°C for 10 min, and cooled to 20°C. The absorbance was measured at 620 nm. The soluble carbohydrate (SC) content (mg·g^{-1}Fw) of the *S. fusiforme* holdfasts was then calculated.

2.11 Statistical analysis

Statistical analysis was performed using SPSS statistical software. Data are expressed as the mean ± standard deviation (mean ± SD), and differences between the treatment and control groups were analyzed using one-way analysis of variance (ANOVA) and Tukey's test. Statistical significance was set at $p< 0.05$.

3 Results

3.1 The regeneration of *S. fusiforme* holdfasts

Figure 1 shows the morphological changes in *S. fusiforme* holdfasts under different exogenous hormone treatments. Compared with normally cultured *S. fusiforme* holdfasts, exogenous addition of NAA at 1.5, 2 mg·L^{-1}, GA_3 at 1.5, 2 mg·L^{-1}, or BAP at 2, 3 mg·L^{-1} could significantly shorten the regeneration time of *S. fusiforme* holdfasts from around 12 days to about 6 days. However, when the hormone concentration was too low or too high, the rate of juvenile regeneration from holdfasts was not significantly promoted. In fact, high hormone concentrations had a significant inhibitory effect on the regeneration of *S. fusiforme* holdfasts.

3.2 Growth and regeneration rates of *S. fusiforme* holdfasts

All NAA concentrations in the range of 1.5, 2 mg·L^{-1} significantly increased the RGR of *S. fusiforme* holdfasts ($p< 0.05$); further, 1.5 mg·L^{-1} of NAA increased the RGR of holdfasts by 118.9% at the end of the culture. In contrast, low concentrations (0.5, 1 mg·L^{-1}) of NAA did not have a significant effect on RGR by the ninth day of incubation ($p< 0.05$; Figure 2A). As shown in Figure 2B, the RGR of holdfasts was not significant ($p< 0.05$) when incubated at or above 2 mg·L^{-1} GA_3. Relative to the other GA_3 concentrations, the highest RGR was observed in holdfasts grown at 1.5 mg·L^{-1}, with an increase of 54.2% over the control group ($p< 0.05$). BAP promoted the growth of holdfasts when its concentration did not exceed 3 mg·L^{-1}, and its promotion effect was the most significant ($p< 0.05$) at 3 mg·L^{-1}, with a 103.8% increase in RGR. However, a high BAP concentration (5 mg·L^{-1}) inhibited the growth of *S. fusiforme* holdfasts after 12 days of incubation (Figure 2C).

NAA at a concentration of 1.5 mg·L^{-1} had the most significant ($p< 0.05$) promotion effect on the RR of *S.*

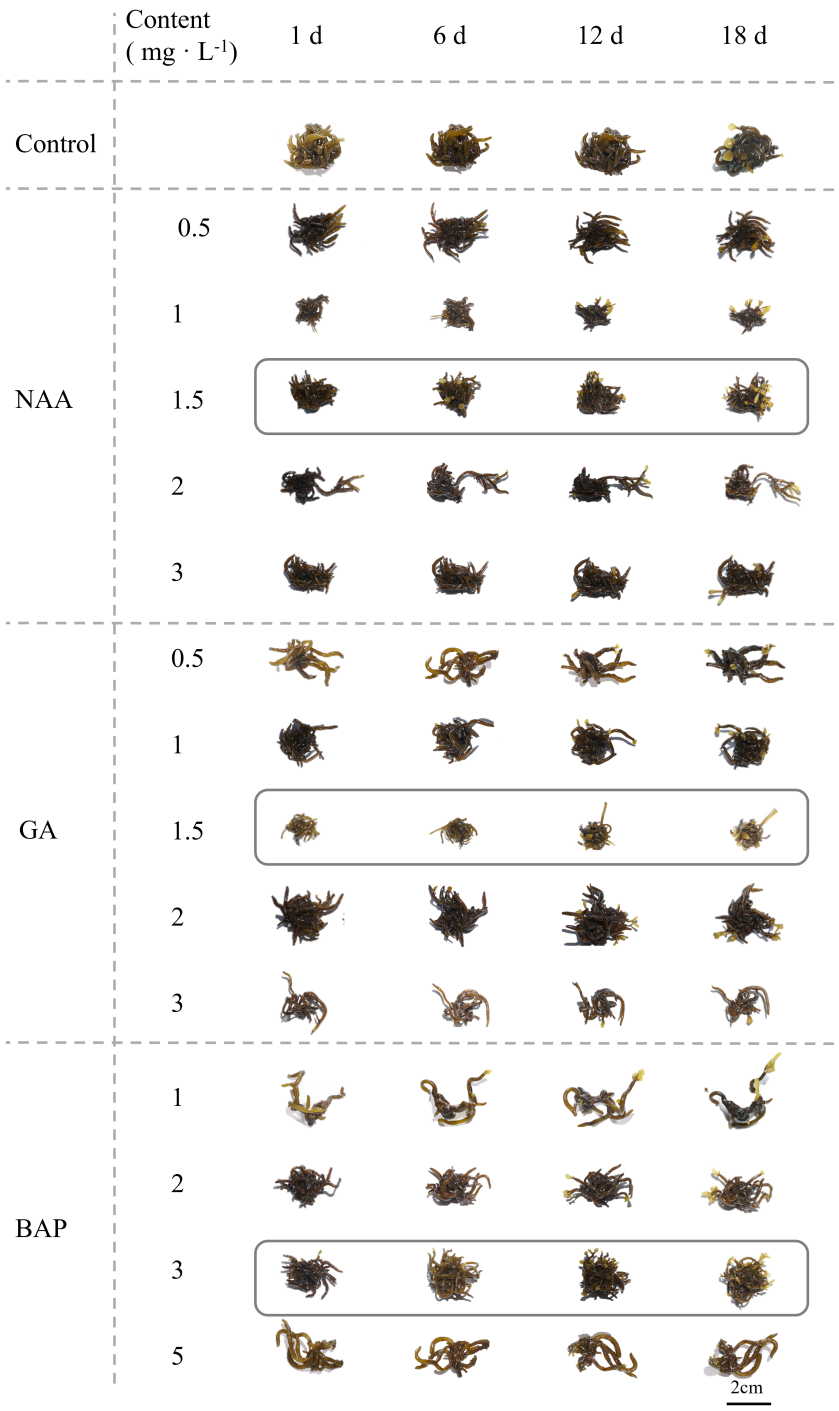

FIGURE 1
Morphological changes in *Sargassum fusiforme* holdfasts with different concentrations of three exogenous hormones applied for different incubation periods.

fusiforme holdfasts, which was 167.4% compared to that of the control (Figure 2D). At a GA_3 concentration of 1.5 mg·L^{-1}, the RR of the holdfasts was 158.8% higher than that of the control ($p< 0.05$; Figure 2E). In contrast, BAP at a concentration of 3 mg·L^{-1} had the most significant promotion effect on the RR of holdfasts, increasing the RR by 123.4% relative to the control ($p< 0.05$; Figure 2F). However, high concentrations of GA_3 and BAP inhibited the regeneration of *S. fusiforme* holdfasts.

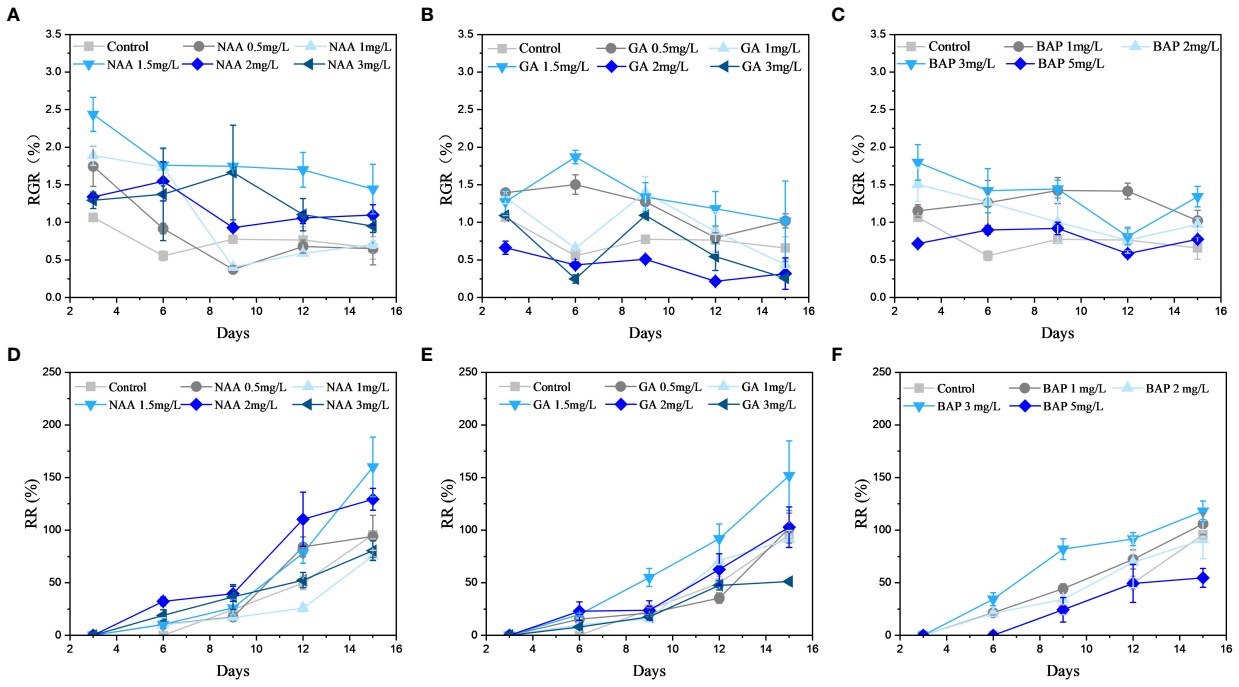

FIGURE 2
Effects of three exogenous hormones on the relative growth rate (RGR) and regeneration rate (RR) of *Sargassum fusiforme* holdfasts. **(A–C)**: relative growth rate; **(D–F)**: regeneration rate.

3.3 Effects of different concentrations of exogenous hormone treatments on the photosynthesis of *S. fusiforme* holdfasts

Exogenous addition of NAA significantly promoted the rETR of holdfasts compared to that of the control, but the difference in the promotion effect between the different concentrations of NAA was not significant ($p< 0.05$; Figure 3A); however, the increase in rETRm values of *S. fusiforme* holdfasts was greater at NAA concentrations of 1.5 and 2 mg·L^{-1} at 26.1% and 26.3% (Figure 3B). As shown in Figures 3C, D, the most significant promotion of photosynthesis in holdfasts was observed when the concentration of GA_3 was 1.5 mg·L^{-1}; and the rETRm values of *S. fusiforme* holdfasts at this concentration increased by 40.6% relative to that of the control ($p< 0.05$). Further, BAP at 2 mg·L^{-1} significantly promoted rETR in holdfasts, and the growth rate of rETRm values of *S. fusiforme* holdfasts at this concentration was 50.1% ($p< 0.05$; Figures 3E, F).

3.4 Photosynthesis and respiration of *S. fusiforme* holdfasts

Exogenous application of NAA significantly promoted the Pm of holdfasts but had a lower effect on Rd; further, the promotion effect was not significantly different among concentrations ($p< 0.05$; Figure 4A). The promotion of the saturated irradiance oxygen evolution was significant when the GA_3 concentration was 0.5, 1.5 mg·L^{-1}, with the maximum promotion effect observed at 1.5 mg·L^{-1}. In contrast, the dark respiration rate of holdfasts was significantly enhanced at GA_3 concentrations of 0.5 mg·L^{-1} and 1.5 mg·L^{-1}, with the strongest respiration at 0.5 mg·L^{-1} ($p< 0.05$; Figure 4B). The Pm of holdfasts significantly increased when the BAP concentration was 2, 3 mg·L^{-1}; the maximum increase was observed at a concentration of 3 mg·L^{-1}. The effect of exogenous BAP application on dark respiration was not significant compared to that in the control and was only slightly higher at 1 and 5 mg·L^{-1} compared to the other treatment groups ($p< 0.05$; Figure 4C).

3.5 The uptake of nitrate-nitrogen by *S. fusiforme* holdfasts

The ability of holdfasts to take up nitrate-N was significantly enhanced when the NAA concentration was 0.5, 2 mg·L^{-1}, and the highest rate of nitrate-N uptake was observed at 1.5 mg·L^{-1} ($p< 0.05$; Figure 5A). Compared to that of the control, NR activity was significantly higher when the NAA concentration was at 1.5 mg·L^{-1} ($p< 0.05$; Figure 5D). The promotion of N uptake

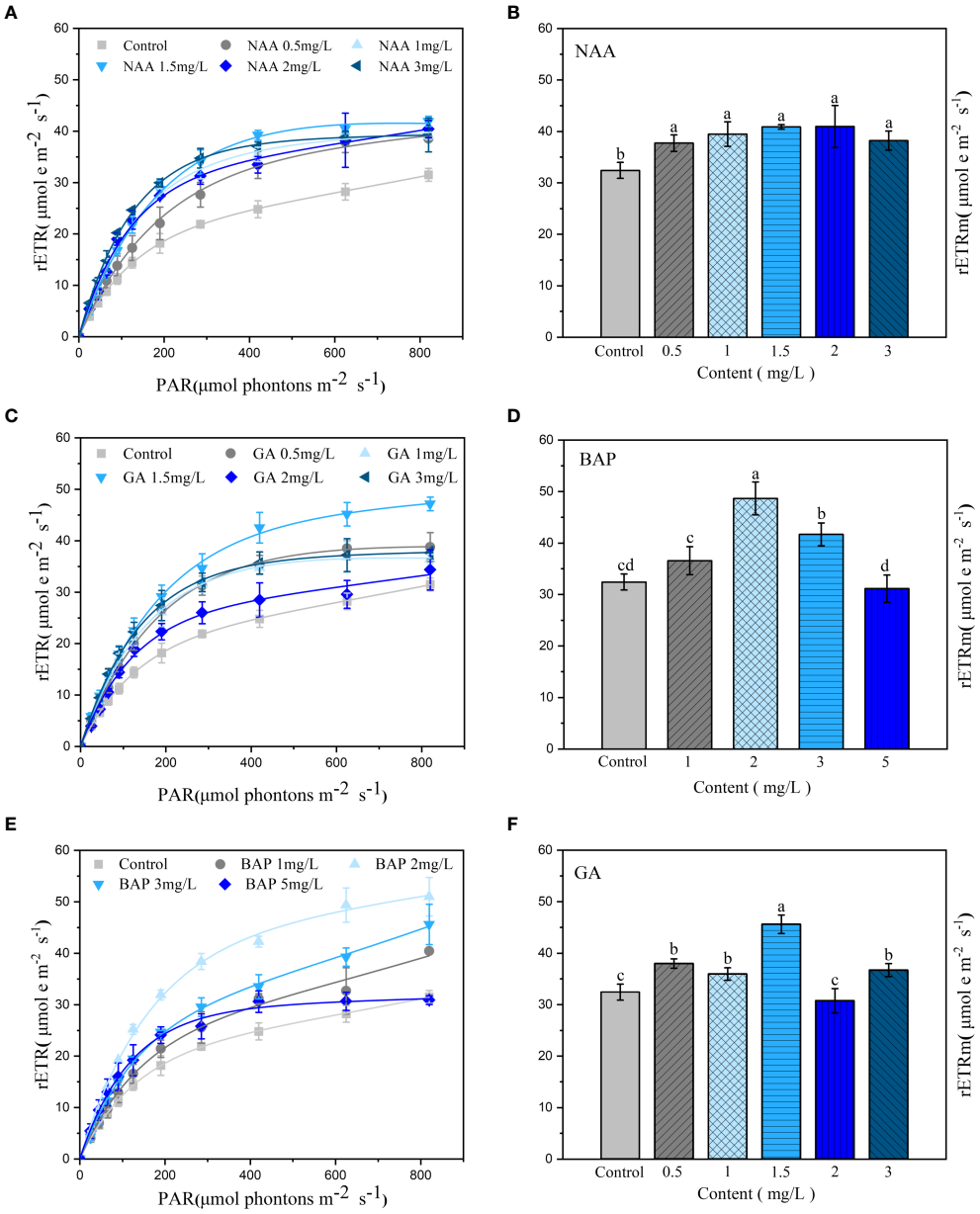

FIGURE 3
Effects of three exogenous hormones on photosynthetic activities in Sargassum fusiforme holdfasts. **(A, C, E)**: relative electron transfer rate (rETR); **(B, D, F)**: the maximum relative electron transfer rate (rETRm). Values are the mean ± standard deviation (n = 3). Different letters indicate significant differences between different culture conditions ($p < 0.05$).

was not significant for low or high GA_3 (0.5, 1 mg·L^{-1}, 3 mg·L^{-1}) treatments and was the most significant only at 1.5 mg·L^{-1}, which showed an increase by 54.3% in the rate of uptake ($p< 0.05$; Figure 5B). As shown in Figure 5E, the NR activity in *S. fusiforme* holdfasts did not change significantly at 0.5 mg·L^{-1} of GA_3; when the GA_3 concentration reached 1, 3 mg·L^{-1} NR activity was significantly enhanced in holdfasts ($p< 0.05$). Exogenous application of BAP significantly promoted the rate of nitrate-nitrogen uptake by holdfasts, with the greatest uptake with the 2 mg·L^{-1} BAP treatment, showing an increase by 57.3% compared to that of the control ($p< 0.05$; Figure 5C). BAP treatment at 1, 3 mg·L^{-1} significantly enhanced the *S. fusiforme* holdfasts and the highest NR activity was observed when the BAP concentration was 3 mg·L^{-1} ($p< 0.05$) (Figure 5F).

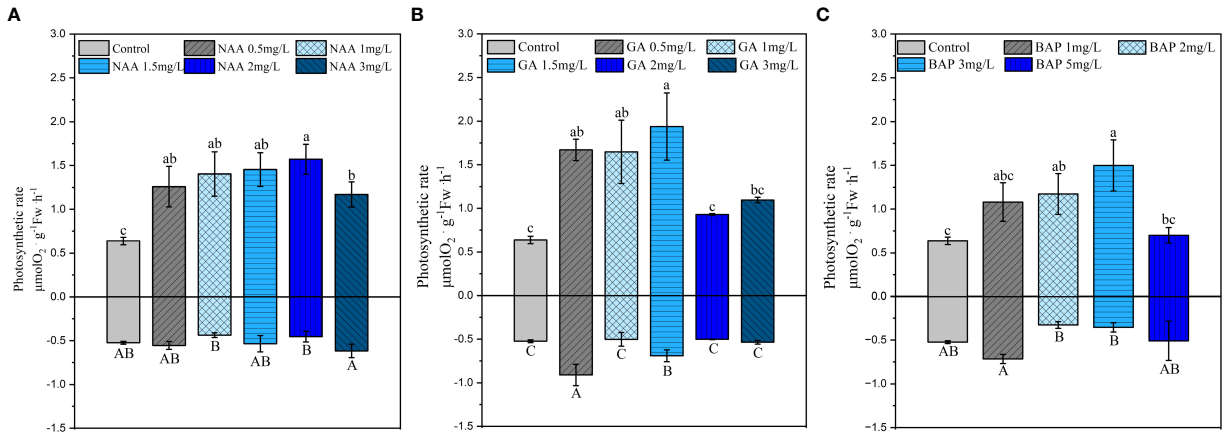

FIGURE 4
Effects of three exogenous hormones on the maximum net photosynthetic rate (Pm) and dark respiration rate (Rd) in Sargassum fusiforme holdfasts **(A, B)**. Values are the mean ± standard deviation (n = 3). Different letters indicate significant differences between different culture conditions ($p < 0.05$).

3.6 The conversion of nitrogen by *S. fusiforme* holdfasts

Exogenously applied NAA treatment significantly enhanced GS activity in *S. fusiforme* holdfasts compared to that of the control, with the strongest GS activity observed in holdfasts treated with 1.5 mg·L^{-1} NAA ($p< 0.05$; Figure 6A). GOGAT activity in the holdfasts was significantly enhanced when the NAA concentration was 1, 1.5 mg·L^{-1} and 3 mg·L^{-1}; the most significant promotion of GOGAT was observed at 1.5 mg·L^{-1} ($p< 0.05$; Figure 6D). Different concentrations of GA_3 showed significant enhancement of GS activity, with the strongest

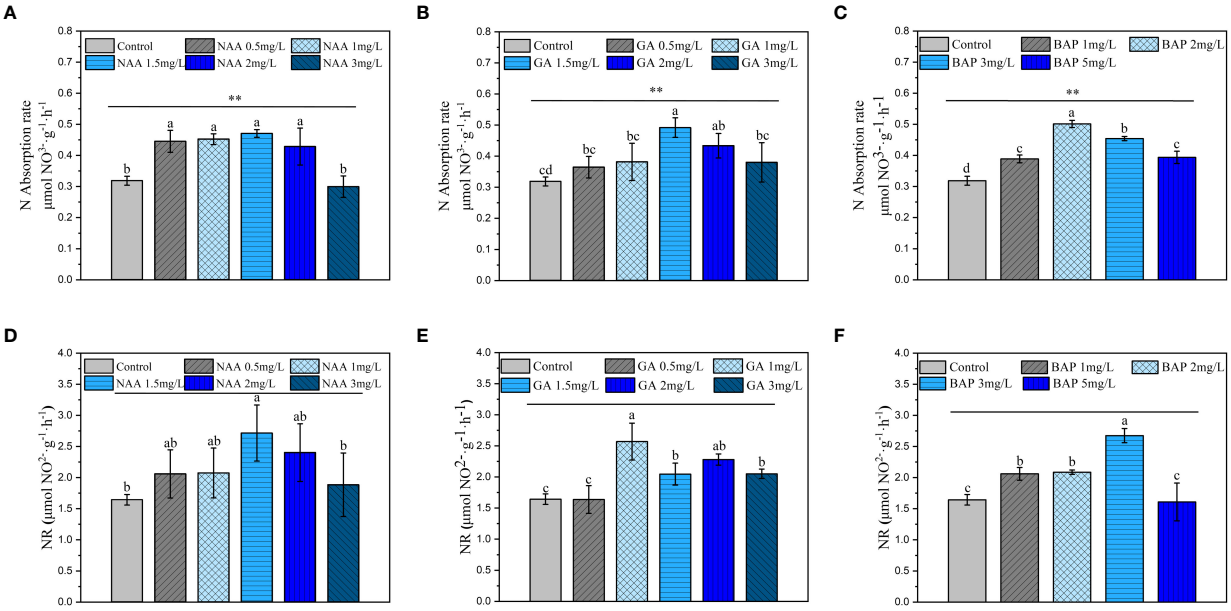

FIGURE 5
Effects of three exogenous hormones on the uptake and reduction of nitrate nitrogen by Sargassum fusiforme holdfasts. **(A–C)**: uptake rate of nitrate nitrogen by holdfasts under different culture conditions; **(D–F)**: nitrate reductase activity in holdfasts under different culture conditions. Values are the mean ± standard deviation (n = 3). Different letters indicate significant differences between different culture conditions ($p < 0.05$). **indicates intergroup variability between different concentrations of exogenous hormones (**$p < 0.01$).

enhancement observed at 1.5 mg·L^{-1} (p< 0.05; Figure 6B). GA_3 significantly enhanced GOGAT activity at 1.5 mg·L^{-1} and 2 mg·L^{-1}, and the most significant effect was observed at 1.5 mg·L^{-1} (p< 0.05; Figure 6E). The GS activity was significantly increased when the BAP concentrations ranged from 1 to 3 mg·L^{-1}, and the strongest effect was observed at 2 mg·L^{-1} (p< 0.05; Figure 6C). As shown in Figure 6F, GOGAT activity was significantly enhanced by different concentrations of BAP, with the most significant (p< 0.05) promotion effect observed at a BAP concentration was 3 mg·L^{-1}.

3.7 Soluble protein and carbohydrate accumulation in *S. fusiforme* holdfasts

When the NAA concentration was greater than 0.5 mg·L^{-1}, the SP content increased significantly in holdfasts, and it further increased with increasing NAA concentration (p< 0.05; Figure 7A). Exogenous application of NAA also significantly increased the content of SC in *S. fusiforme* holdfasts compared to that in the control, but the difference between different concentrations was not significant (p< 0.05; Figure 7D). Different concentrations of GA_3 significantly increased the content of SP and SC in holdfasts, with the highest SP and SC contents observed at the 1.5 mg·L^{-1} GA_3 treatment (p< 0.05; Figures 7B, E). BAP treatment significantly increased the SP content in *S. fusiforme* holdfasts in a dose-dependent manner (p< 0.05; Figure 7C). The SC content in holdfasts was significantly increased at BAP concentrations of 2, 3 mg·L^{-1}, with the highest SC content observed at 3 mg·L^{-1} BAP (p< 0.05; Figure 7F).

4 Discussion

The type and content of PGRs remarkably affect the photosynthetic rate and pigment content of seaweeds, and the appropriate concentrations of phytohormones promote the photosynthetic rate, carbonic anhydrase activity, and C/N ratio of seaweeds (Wei et al., 2020). Light and growth factors regulate each other to maintain a balance for normal plant growth (Xu et al., 2018). In the present study, exogenous application of NAA significantly enhanced photosynthesis in holdfasts, but the difference between different concentrations examined in this study was not significant.

After NO_3^- - N is absorbed by algae, it continues to be reduced to NH_4^+- N by NR reduction and then enters the GS/GOGAT cycle to synthesize nitrogenous compounds, such as proteins and nucleic acids (Balotf et al., 2016; Liu et al., 2022). In

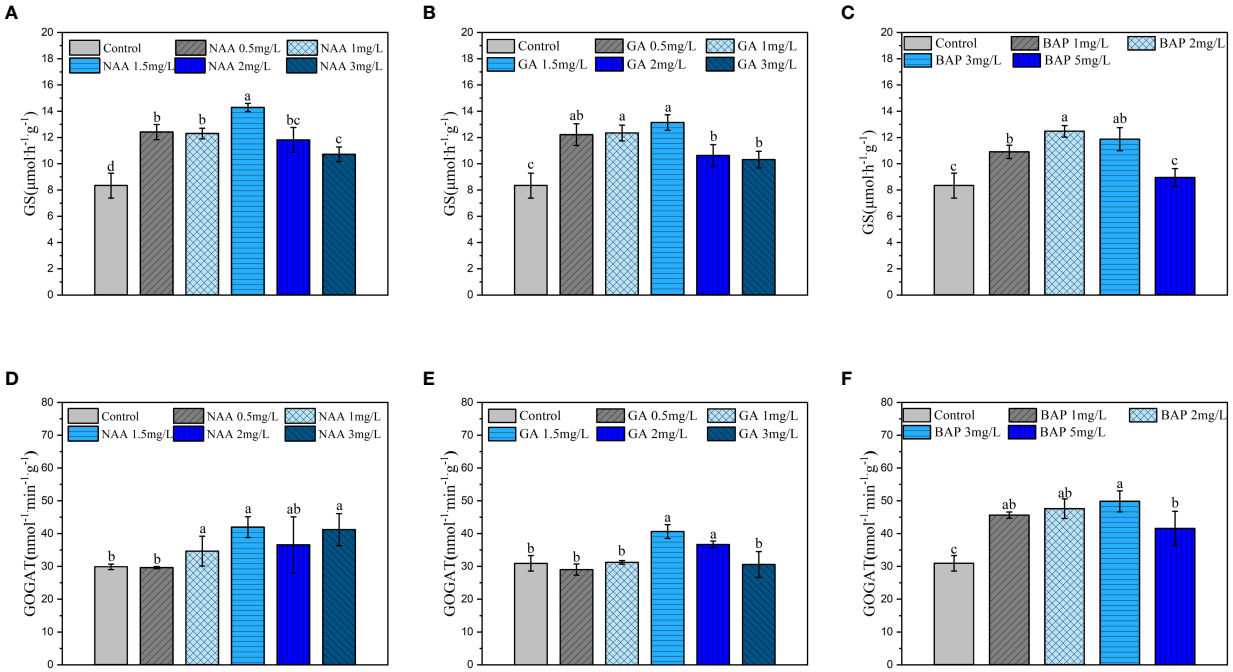

FIGURE 6
Effects of three exogenous hormones on nitrogen assimilation by Sargassum fusiforme holdfasts. **(A–C)**: glutamine synthetase (GS) activity in holdfasts under different culture conditions; **(D–F)**: glutamate synthase (GOGAT) activity in holdfasts under different culture conditions. Values are the mean ± standard deviation (n = 3). Different letters indicate significant differences between different culture conditions (p < 0.05).

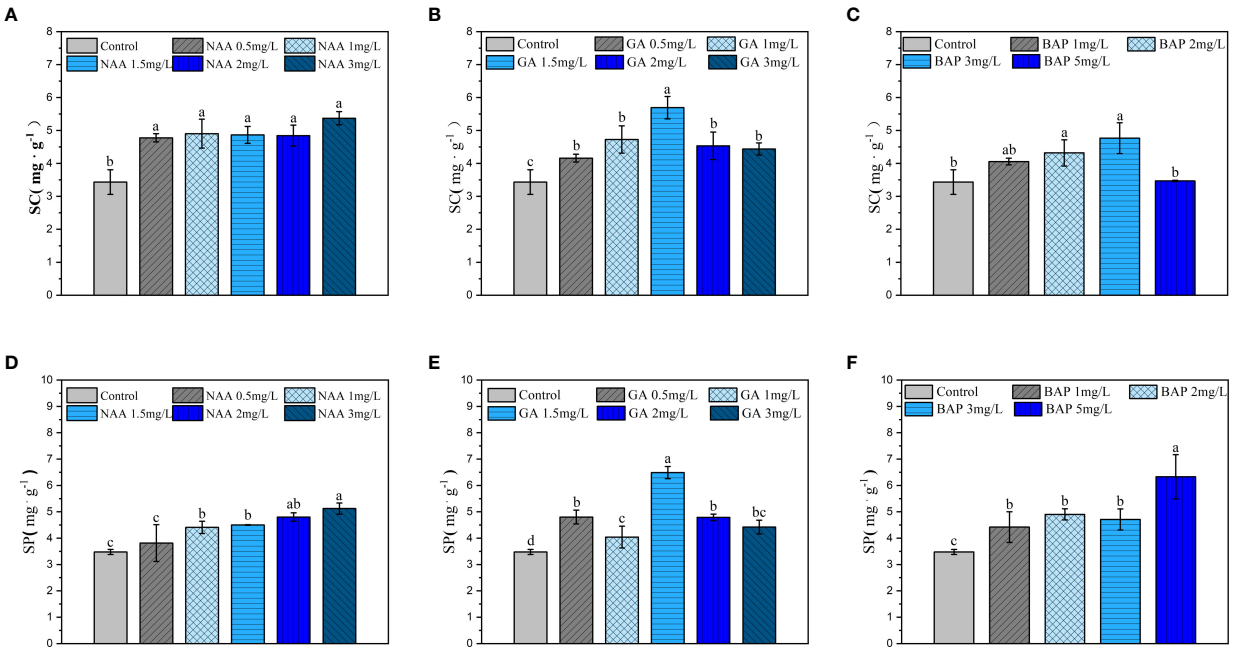

FIGURE 7
Effects of three exogenous hormones on the accumulations of soluble protein and carbohydrate in Sargassum fusiforme holdfasts. **(A–C)**: soluble protein contents in the holdfasts under different culture conditions; **(D–F)**: soluble carbohydrate contents in holdfasts under different culture conditions. Values are mean ± standard deviation (n = 3). Different letters indicate significant differences between different culture conditions ($p < 0.05$).

this study, exogenous addition of NAA significantly promoted the nitrogen uptake and transformation capacity of *S. fusiforme* holdfasts with the maximum promoting effect observed at 1.5 mg·L^{-1}. Compared with that of GOGAT, NAA had a more significant enhancing effect on GS activity in holdfasts. This indicates that NAA treatment at 0.5, 2 mg·L^{-1} significantly promoted the rate of nitrate nitrogen uptake and the ability to convert it to amino acids in *S. fusiforme* holdfasts, with significant differences in the effects between concentrations only observed in the results of GS activity assays. NO_3^- and NAA help increase the uptake of N, P, K, and Ca^{2+} in some higher plants, and exogenous application of NAA during tissue culture is known to increase the activities of key enzymes in the nitrogen assimilation pathway, including NR, GS, and GOGAT (Srivastava and Dwivedi, 2003; Tavakoli Hasanaklou et al., 2021). These conclusions are highly consistent with our experimental results.

In this study, exogenous addition of GA_3 promoted photosynthesis in *S. fusiforme* holdfasts with maximum promotion at a concentration of 1.5 mg·L^{-1}. GA is involved in light signal perception, such as photosensitive pigment pathways during seed regeneration, photomorphogenesis during yellowing and stem elongation, and photoperiodic regulation during development (Wu et al., 2019; Hu et al., 2022). During *S. fusiforme* holdfast regeneration, GA_3 may achieve photosynthesis-promoting effects by regulating the photoperiod and light sensitivity. In a previous study, addition of 20 μM GA_3 to the *in vitro* medium of *Strelitzia reginae* conidia embryos increase nitrate reductase activity (Figueiredo et al., 2021). In this study, exogenous application of 1.5 GA_3 mg·L^{-1} significantly promoted the uptake and transformation of nitrate-nitrogen by *S. fusiforme* holdfasts.

Exogenous addition of different BAP concentrations increased the relative electron transfer rate of photosystem II in *S. fusiforme* holdfasts. The photosynthesis of holdfasts was sensitive to BAP concentration during the regulated process, and its effect decreased with increasing or decreasing concentrations, showing the most significant promotion effect at 2 mg·L^{-1}. Exogenous addition of cytokinin has been shown to modulate the inhibitory effect of physical stress on the PSII operating efficiency in maize leaves, thus improving gas exchange; further, BAP-treated plants have higher ΦPSII and ETR than untreated plants under stress conditions (Acidri et al., 2020; Wang et al., 2022). Moreover, adenine-type cytokinins have been shown to enhance the activities of carbon-metabolizing and nitrogen-assimilating enzymes in *Chlorella vulgaris* (Piotrowska and Czerpak, 2009). In the present study, exogenous application of 1–3 mg·L^{-1} BAP significantly enhanced the activity of nitrogen-

assimilating enzymes in *S. fusiforme* holdfasts, suggesting that cytokinins may have similar regulatory mechanisms in macroalgae and microalgae.

In general, accumulation of soluble proteins in plants is strongly influenced by nitrogen uptake and utilization (Chen et al., 2017a; Chen et al., 2018; Tian et al., 2022). When the RR of *S. fusiforme* holdfasts was higher, the synthesized SP was used more for the growth of regenerated seedlings, despite the fuller uptake and utilization of nitrogen (Xu et al., 2022b). In the present study, the SP content in holdfasts was related to enzymatic activity during nitrogen conversion and utilization, as well as to the RR (juvenile growth) of *S. fusiforme* holdfasts under these conditions. The SC content was related to plant photosynthesis and respiration (Chen et al., 2017b; Chen et al., 2019; Zhang et al., 2020). In this study, 5 $mg{\cdot}L^{-1}$ of BAP enhanced the dark respiration of holdfasts, leading to the depletion of carbohydrates accumulated by photosynthesis. In contrast, the promotion of photosynthesis by other hormones and concentrations in the holdfasts was more consistent with the changes that increased the SC content.

Overall, appropriate concentrations of NAA, GA_3, and BAP could promote faster and greater regeneration of juveniles from *S. fusiforme* holdfasts. Combined with other relevant physiological indicators, the most suitable concentrations of NAA, GA_3, and BAP for holdfast regeneration were 1.5 $mg{\cdot}L^{-1}$, 1.5 $mg{\cdot}L^{-1,}$ and 3 $mg{\cdot}L^{-1}$, respectively. Therefore, the above concentrations of exogenous hormones can be added in practice in the early stages of culture to shorten the culture time of juveniles' regeneration and to obtain more regenerated juveniles for improve the economic efficiency. Moreover, although the high concentrations of GA_3 and BAP inhibited the regeneration from *S. fusiforme* holdfasts during the culture process, they did not significantly inhibit the physiological activities of holdfasts by the end of the culture. This property provides a new perspective for regulating the propagation of *S. fusiforme* holdfasts.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding authors.

Author contributions

LL and BC contributed to complete the experiments, investigate and analyze the data, and wrote original draft of the paper. XZ, LG, GP contributed to sample and process the experiments. ZM contributed to revise the paper. MW and BC contributed to conceptualize, supervise, review and edit the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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