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RESEARCH

Cultivation of *Gongolaria barbata* (Fucales, Phaeophyceae) with a seaweed-derived biostimulant in order to improve photophysiological fitness and promote fertility to advance the restoration of marine macroalgal forests

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Abstract

As a result of several anthropogenic factors, *Cystoseira* sensu lato forests have declined or become regionally extinct in many coastal regions of the Mediterranean. Given the low natural recovery of lost populations, research efforts have been encouraged to develop sustainable and efficient restoration of macroalgal forests on a large scale. By promoting growth and fertility of collected thallus branches under controlled laboratory conditions, the availability of seedlings for restoration could be ensured without jeopardizing natural populations. Here we investigated the effect of a commercial algal biostimulant (AlgatronCifo®) on the photophysiology, growth and fertility of *Gongolaria barbata* (Stackhouse) Kuntze (Fucales, Phaeophyceae). In a factorial laboratory experiment, two different temperatures (10 ºC and 14 °C) and two culture media [i.e. seawater (SW) and Algatron (AT)] were tested. The photosynthetic performance of *G. barbata* doubled after three weeks of culture with AT, while it decreased by 25% when cultivated in SW. The highest photosynthetic performance and growth were achieved at 14ºC with AT, where fertile receptacles also developed, followed by seedling settlements. The thalli cultured in AT had similar or better photosynthetic performance than the initial control thalli. AT-cultured thalli had a greater ability to quench energy via photochemical pathways (q_P) than those from the SW, which on the contrary, had higher levels of non-photochemical responses (q_N, NPQ_{max}). This limited photosynthetic performance was probably linked to the higher P-limitation experienced under that treatment. The algal biostimulant enhanced the physiological performance and induced fertility of *G. barbata*, demonstrating its valorization potential and setting a new path for improved restoration applications.

Keywords Algal biostimulant · Ecological restoration · *Gongolaria barbata* · PAM fluorometry · Phaeophyceae · Photophysiology

Introduction

Cystoseira sensu lato (Fucales, Phaeophyceae) species are the main foundation species of Mediterranean algal forests (Fabbrizzi et al. 2020), listed in several international agreements for the conservation of marine species and habitats (e.g., Bern Convention, Barcelona Convention, Directive 92/43/EEC, European Red List of Habitats). However, as a result of multiple anthropogenic stressors (e.g., coastal urbanization, eutrophication, sediment input and overgrazing), these species and their habitats have been steadily declining in many coastal regions and are now critically endangered (Mangialajo et al. 2008; Falace et al. 2010; Vergés et al. 2014; Thibaut et al. 2015; Blanfuné et al. 2016;

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Valdazo et al. 2017; Mancuso et al. 2018). This trend of degradation is fueling intensive research efforts aimed at implementing effective measures to replenish declining populations of *Cystoseira s.l.* or restore lost forests (Falace et al. 2018; Verdura et al. 2018; De La Fuente et al. 2019; Orlando-Bonaca et al. 2021a, 2022; Savonitto et al. 2021).

Recruitment-enhancement, both ex situ and in situ, are both more sustainable methods than transplanting adult thalli (Falace et al. 2006) in order to restore macroalgal forests as they rely on harvesting only reproductive, fertile thallus branches which avoids the complete removal of adult plants from wild donor populations (Falace et al. 2018; Verdura et al. 2018). While the *in-situ* technique simulates the recruitment process by fixing fertile branches directly at the restoration site (Verdura et al. 2018), the *ex-situ* technique consists of cultivating seedlings in mesocosms to maximize recruitment and survival by setting the optimal culture conditions of temperature, light and nutrients (Falace et al. 2018; De La Fuente et al. 2019; Savonitto et al. 2021; Orlando-Bonaca et al. 2022).

However, scaling up restoration activities requires multiple and reiterative harvesting of fertile branches to obtain an adequate number of recruits. This could jeopardize the survival of donor populations and reduce their reproductive potential. On the other hand, the phenology of natural populations, their reproductive potential and consequently their restoration success are seriously threatened by increasingly frequent marine heat waves (Bevilacqua et al. 2019; Savonitto et al. 2019). Finally, the reproductive season in most species is limited to a few months and is increasingly unpredictable and altered due to climate change, as observed in the northern Adriatic after a heatwave (Bevilacqua et al. 2019; Savonitto et al. 2019). A possible solution to avoid burdening donor stocks and to decouple the availability of recruits from the natural reproductive cycle is to cultivate *Cystoseira s.l.* by inducing sexual maturity to ensure a seedling reservoir. However, seaweed farming is not an easy task as many problems may arise, such as growth loss, reduced plant quality, diseases, and competition from endo- or epiphytes, resulting in lower productivity and a shortage of seedlings (Hurtado and Critchley 2018; Jiksing et al. 2022).

Attempting to overcome these issues, seaweed-derived biostimulants have been used to increase the survival, growth and stress tolerance of a number of selected macroalgae, including brown (Hurtado and Critchley 2018, 2020; Umanzor et al. 2019, 2020a, b, 2022; Ali et al. 2021; Jiksing et al. 2022; Han et al. 2023). Macroalgal biostimulants are emerging as sustainable biological growth promoters and are increasingly used to improve agronomic production, plant growth and health (Crouch and Van Staden 1993; Battacharyya et al. 2015; Trivedi et al. 2018; Hurtado and Critchley 2020; Samuels et al. 2022). Their modes of action are not yet fully understood, but several studies have shown that this type of products has several beneficial effects on plants due to their broad spectrum of constituents (i.e. macro- and microelements, amino acids, hormones, phenolic compounds and saccharides) (Khan et al. 2009; Stirk et al. 2020; Ali et al. 2021; Sujeeth et al. 2022). The biostimulatory algal constituents promote natural processes for efficient nutrient uptake and utilization, chlorophyll content and photosynthesis, stress resistance, root development and also trigger early flowering and seed germination (Blunden and Wildgoose 1977; Crouch and Van Staden 1993; Arthur et al. 2003; Kumari et al. 2011; Spann and Little 2011; du Jardin 2015; Martynenko et al. 2016; Santaniello et al. 2017; Van Oosten et al. 2017; Zhang et al. 2019; Ali et al. 2021; Shukla et al. 2022; Sujeeth et al. 2022).

While the use of biostimulants from algae is currently gaining traction in marine agronomy (Hurtado and Critchley 2020; Jiksing et al. 2022), their application in the context of ecological restoration has not yet been explored. Therefore, the present study investigated the effects of a commercial algal biostimulant AlgatronCifo® derived from *Macrocystis pyrifera* (Linnaeus) C. Agardh (Laminariales, Phaeophyceae) on the photophysiology, growth and fertility of *Gongolaria barbata* (Stackhouse) Kuntze (= *Cystoseira barbata*) (Fucales, Phaeophyceae) to advance *ex-situ* restoration of macroalgal forests. We hypothesize that the algal biostimulant could lead to higher photosynthetic performance as compared to thalli grown in seawater, along with enhancement of photochemical pathways of photosynthesis in *G. barbata* and, thus, to promote its growth and fertility.

Materials and methods

Sampling site

The Gulf of Trieste is a shallow (max depth 25 m), semi-enclosed continental shelf area in the northeastern part of the Adriatic Sea with highly variable oceanographic features (Cozzi et al. 2020). The area is characterized by a marked seasonal cycle of seawater temperature (from winter minima of 8.0 °C to summer maxima of 28.4 °C) and strong salinity gradients (from 24.0 to 38.3‰) (Malačič et al. 2006; Kralj et al. 2019). The balance of nutrients and organic matter in the Gulf of Trieste are influenced by river discharges, especially by the Isonzo River, as well as by wastewater discharges and benthic fluxes (Cozzi et al. 2020). The trophic status of the Gulf also depends on the prevailing circulation patterns (Cibic et al. 2022).

The sampling site was located close to the Marano and Grado Lagoon, which is characterized by high seasonal and spatial variability of nitrogen, especially in the form of $N\text{-}NO_3^-$, and P limitation, as shown by the high DIN:SRP ratio (Acquavita et al. 2015). In situ nutrient concentrations

from the area of study are summarized in Supplementary Information (Table S1).

Gongolaria barbata has declined in much of the Gulf of Trieste over the last three decades (Falace and Bressan 2003; Falace et al. 2005, 2010) and is now found only at a few sites with scattered populations (Orlando-Bonaca et al. 2021b; Savonitto et al. 2021).

In early November 2021, about 50 primary branches of *G. barbata* about 20 cm long (Fig. 1a) were collected on the beach near Grado (45°40′55.5 "N 13°26′04.2 "E) (Fig. 1b). Thalli collected were sterile and without receptacles as *G. barbata* normally reproduces from March to May in the northern Adriatic (Falace et al. 2006; Savonitto et al. 2021; Orlando-Bonaca et al. 2022). Fronds were transported in buckets of seawater in the dark and cold (4 °C) to the Phycological Laboratory of the University of Trieste within 1 h of collection.

Experimental set-up

In the laboratory only specimens without visible damage to the thallus or signs of degradation and depigmentation were selected for the experiment, then carefully wiped with paper towel and rinsed with filtered seawater (0.22 µm Durapore membrane filters, Merck Millipore Ltd) to remove visible epiphytes. For acclimation, thalli were maintained in filtered seawater (SW) for 48 h at 14 °C in tanks with a light intensity of 125 µmol photons m^{-2} s^{-1} (LED lamps, AM366 Sicce USA Inc., USA) and a photoperiod of 15:9 light:dark (Orlando-Bonaca et al. 2022). Light irradiance was measured using a LI-COR LI -190/R photometer (LICOR-Biosciences, USA).

After acclimatization (T0), a factorial laboratory experiment was performed in two environmentally controlled rooms to test two different temperatures and two culture media. Temperatures were set at 10 ºC (i.e., temperature measured in the field) and 14 °C (i.e. the mean temperature at which brown alga normally reproduces in the study area; Orlando-Bonaca et al. 2022) (Fig. 2). The culture media tested were filtered seawater (SW) and a solution of filtered SW enriched with the commercial biostimulant derived from *Macrocystis pyrifera* AlgatronCifo® (AT) (Cifo S.p.A., San Giorgio di Piano, Bologna, Italy. https://www.cifo.it/en/product/home-and-garden/hg-products/nutrition-and-beauty/specialities/pure-energy-for-all-plants/) at the concentration recommended by the manufacturer for foliar and fruiting treatments on land plants (i.e., 4.5 mL L^{-1}). The nutrient concentrations of the culture media used in this study (ie., SW and AT) are given in Table 1, and the concentrations of nutrients and major components of pure Algatron are given in Supplementary Information (Table S1). Furthermore, M. pyrifera extracts contain alginate, phytohormones and a variety of mineral nutrients, such as magnesium, molybdenum, calcium, phosphorus, iron, zinc and boron (Iparraguirre et al. 2023).

For each treatment, nine replicate flasks were filled with 2 L of culture medium, and each flask contained a thallus frond that was cultured for three weeks (T3) (Fig. 2). The medium in each flask was renewed twice a week to prevent possible nutrient limitation. Aerators provided oxygenation of the medium.

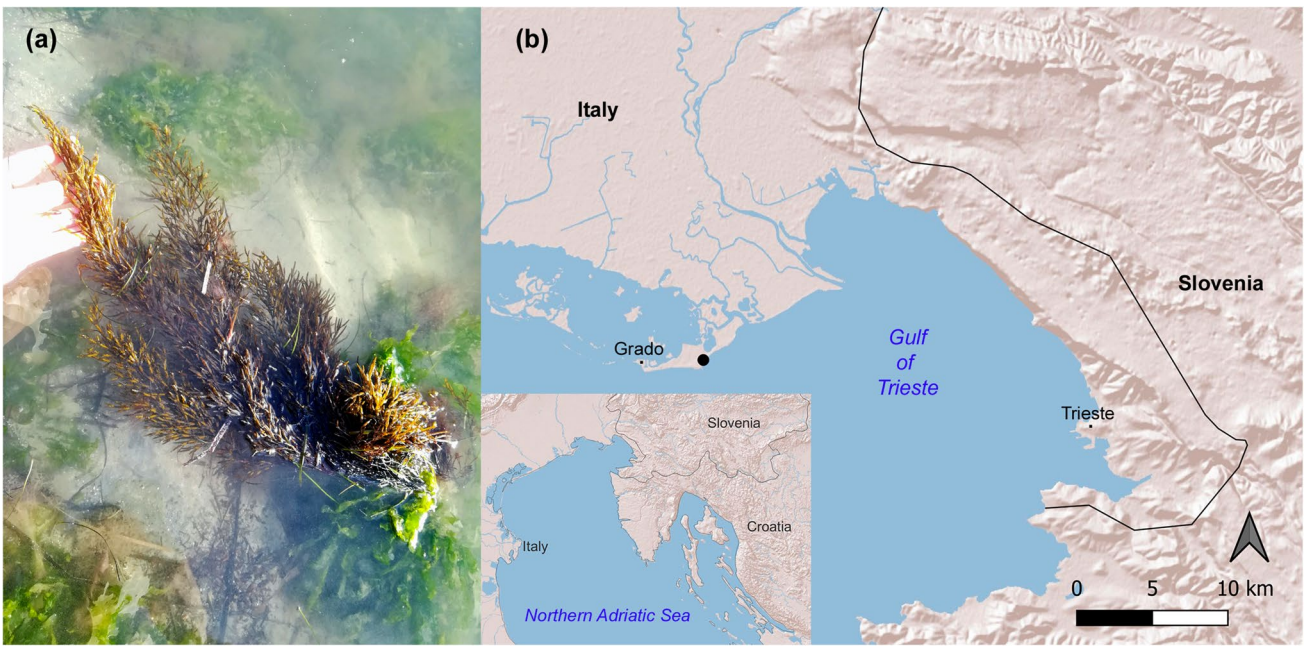

Fig. 1 **a)** *Gongolaria barbata* at the sampling site; **b)** Sampling site (Gulf of Trieste, Northern Adriatic)

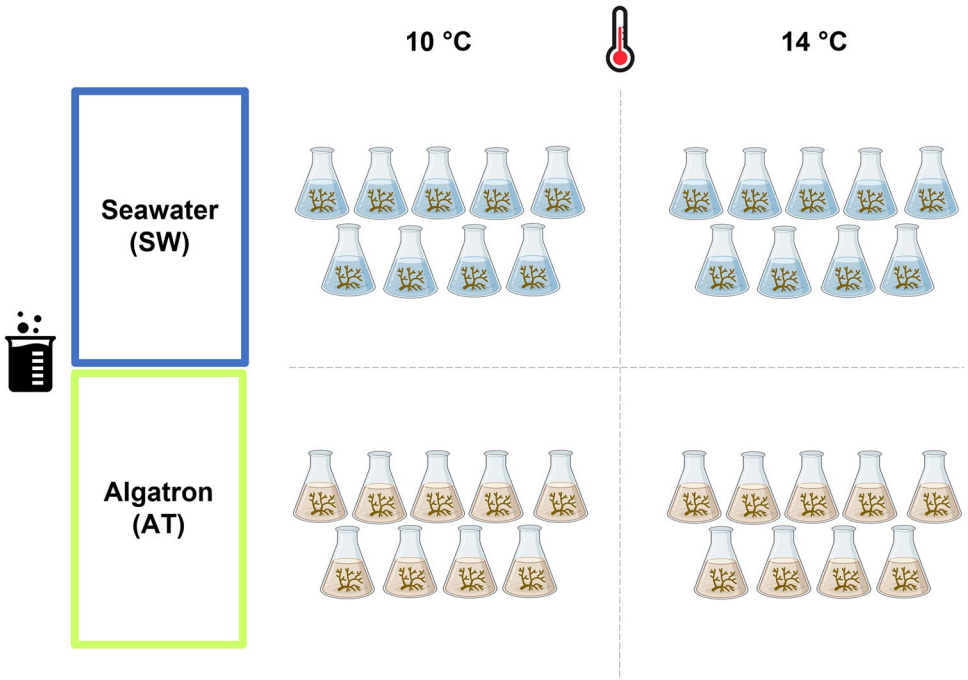

Fig. 2 Experimental design: for each treatment, nine replicate 2-L flasks, each with a primary branch, were used to test the effects of temperature and culture medium on the performance of the culture of *Gongolaria barbata*

Table 1 Culture media used in the study

	Seawater (SW)*		Seawater enriched with Algatron (AT)	
	µg L^{-1}	µM	µg L^{-1}	µM
TN	137.00	10	530.60	37.90
Urea-NO_2^-			0.03	0.0005
N-NH_4^+	6.54	0.4	373.50	20.75
N-NO_3^-	52.54	0.85	53.30	0.86
TP	6.13	0.20	7.03	0.23
PO_4^{3-}	1.00	0.01	1.97	0.02
DIN:SRP molar ratio		121		1044
TN:TP molar ratio		50		

*(mean values for 2021—data provided by ARPA FVG and are derived from the monitoring carried out by the Agency for the purpose of classifying the regional water bodies pursuant to Legislative Decree 152/2006 as amended (transposition of the Water Framework Directive 2000/60/EC)

Growth and fertility induction

At T0 and T3, five fronds from each treatment were carefully blotted-dry to remove excess water, and wet biomass (WB) was measured. Growth was calculated as relative growth rate (RGR) according to the following equation (Lüning 1990):

$$RGR\left(day^{-1}\right) = \frac{\left(LnWBT_3 - LnWBT_0\right)}{T_3 - T_0} \quad (1)$$

where WBT_0 and WBT_3 are the initial and final wet biomass and T is the experimental duration (21 days).

Throughout the experiment branch tips were observed for possible reproductive structures. When present, the receptacles were examined with a stereo microscope (Leica, MZ 6) and photographed with a Nikon Coolpix 4500 camera. At T3, fertile receptacles were detached from branches cultured at 14 °C in AT (i.e., the only condition under which they developed) to check whether they fertilized and produced viable zygotes. Receptacles were stored according to the protocol for *ex-situ* cultivation of *G. barbata* (Savonitto et al. 2021; Orlando-Bonaca et al. 2022) for 24 h at 4 °C in order to induce gamete release, and then randomly distributed to three Petri dishes, each containing nine fertile receptacles, and filled with AT. The receptacles were removed from the Petri dishes after 24 h. After fertilization (AF), the zygotes were cultured for 4 weeks (T5). The number of embryos per Petri dish was determined at 2 weeks AF (T4) and at T5. The development of the embryos was photographed with a Canon Powershot G9 on an inverted microscope (Leica, DM IL LED).

Photosynthetic performance

In vivo chlorophyll-*a* fluorescence (Chl_aF) of photosystem II (PSII) allowed the assessment of the photosynthetic activity (Krause and Weis 1984; Murchie and Lawson 2013) of the specimens of *G. barbata*. Chl_aF was measured on the apical part of fronds (10 cm) using a PAM-Imaging Fluorometer Open FluorCam (Photon Systems Instruments, Czech

Republic). Photosynthetic efficiency was measured on three randomly selected fronds (i.e. Excel function RANDOM) at the end of acclimation (T0), after 7 days (T1), after 14 days (T2) and after 21 days (T3). To avoid signal overflow, the shutter time and sensitivity of the charge-coupled device (CCD) camera were set to 1 and 10 respectively. Each frond was placed at a constant distance of 17.5 cm below the camera lens, and the lamps were placed at an angle of 45° to the center of the measurement area. Prior to the measurements, each frond was dark-adapted for 20 min to allow complete oxidation of the PSII reaction centers.

The basal fluorescence (F_0) was measured. Then a saturating pulse of actinic light (1990 µmol photons m^{-2} s^{-1}, 0.8 s) was administered to induce maximum fluorescence (F_m). The maximum quantum yield was calculated after equation (Maxwell and Johnson 2000):

$$\frac{F_v}{F_m} = \frac{F_m - F_0}{F_m} \tag{2}$$

Rapid Light Curves (RLCs) consisted of 8 actinic light steps at the following intensities 29, 88, 147, 206, 324, 442, 795, 1149 µmol photons $m^{-2} \cdot s^{-1}$ and lasting 60 s each. Irradiance period was 60 s to ensure the minimum (steady-state) fluorescence in actinic light (F_t) as in Nielsen and Nielsen (2008). A saturating light pulse of 4040 µmol photons m^{-2} s^{-1} was applied at the end of every step to determine the minimum and maximum fluorescence (F and F'_m), and thus the effective quantum yield of PSII according to equation (Genty et al. 1989):

$$Y(II) = \frac{\Delta F}{F'_m} = \frac{F'_m - F}{F'_m} \tag{3}$$

The relative electron transport rate (rETR) was calculated by multiplying Y(II) by the respective PAR values at each light step according to the equation (Genty et al. 1989):

$$rETR = Y(II) \times PAR \tag{4}$$

RLCs with rETR as a function of PAR were fitted to the Platt et al. (1980) model using the PHYTOTOOLS package (Silsbe and Malkin 2015) in R (R Core Team 2022). The initial slope alpha (α), the light saturation coefficient (E_k) and the maximum relative electron transport rate ($rETR_{max}$) were calculated from each curve following equation:

$$rETR = rETR_m \times [1 - exp(\frac{-\alpha \times PAR}{rETR_m})] \tag{5}$$

For each light step, the non-photochemical quenching (NPQ) of Chl_aF was also calculated using the following equation:

$$NPQ = \frac{F_m - F'_m}{F'_m} \tag{6}$$

To obtain the values of the maximum non-photochemical quenching (NPQ_{max}),which represents the maximum thermal energy dissipation (Joliot and Johnson 2011), the NPQ *vs.* PAR curves were fitted according to Serôdio and Lavaud (2011), using "*phytotools*" package version 1.0 (Silsbe and Malkin 2015) in R (R Core Team 2022).

Additionally, photochemical and non-photochemical quenching was estimated from RLC data. The proportion of opened reaction centers, and thus the efficiency of the photochemical pathway during the energy dissipation by activated Chlorophyll *a* was described by q_P (Maxwell and Johnson 2000) and calculated after Schreiber et al. (1986). Non-photochemical quenching represents the energy dissipation different from the fluorescence and the photochemical pathway, being in relation with heat dissipation (Maxwell and Johnson 2000) and was calculated as q_N (Schreiber et al. 1986) and NPQ (Bilger and Björkman 1991).

Additionally, the relationships between photochemical (q_P) and non-photochemical quenching (q_N) of thalli of *G. barbata* against E/E_k were compared among treatments. Parameters q_P and q_N were represented against the ratio E/E_k to analyze them at the limiting ($E/E_k < 1$), optimal ($E/E_k = 1$) and saturating ($E/E_k > 1$) range of irradiances for RLCs (Burdett et al. 2019). Response curves of quenching parameters to E/E_k ratio were fitted by non-linear regression to a standard dose–response curve (four-parameter logistic model), which is used when X values are logarithms of doses, following the equation:

$$Y = Bottom \frac{(Top - Bottom)}{1 + 10^{[\left(loglog\left(\frac{E}{E_k}50\right) - X\right) * Hillslope]}}$$

where *X* is the log of the ratio E/E_k [the ratio between incident irradiance (PAR = E) and the saturating irradiance for photosynthesis derived from the rapid light curves (E_k)]; *Y* is the quenching response (photochemical-q_P, or non-photochemical q_N); *Top* and *Bottom* are the plateaus from the logistic model in the same units than quenching parameters; $logE/E_{k50}$ is the ratio at which 50% of maximum quenching response is reached (unitless); and *HillSlope* is the slope factor (unitless), which represent the highest rate of change of the quenching parameters in the model.

Statistical analyses

Analysis of Variance (ANOVA) was performed to test for the effect of medium, temperature and time of exposure on F_v/F_m, $rETR_{max}$, α_{ETR} and NPQ_{max}. The design for the analysis consisted of three factors: Time (Ti, fixed, 3 levels), Medium (Me, fixed, 2 levels, crossed), and Temperature (Te, 2 levels, crossed), with $n = 3$ for each combination of factors. Significant interaction terms were examined by performing a post hoc pairwise *t*-test. The assumption of

normality of response variables was tested with the Shapiro–Wilk test. Cochran's *C*-test was used to test for the assumption of homogeneity of variances prior to analysis (Underwood 1997). For all response variables except F_v/F_m, the assumptions of normal distribution and variance homogeneity were verified. In this last case, non-normality and variance heterogeneity persisted after transformation. Therefore, data were analyzed through a permutational multivariate analysis of variance (PERMANOVA: Anderson 2001) based on Euclidean distance, with 5,000 permutations. This analysis returns the classical univariate F statistic, and it can be used to substitute univariate ANOVA, but it does not make any assumption on data distribution and is robust to variance heterogeneity for experiments with balanced designs (Anderson 2017).

Two-way ANOVA was used to test for the effects of culture medium (two levels: SW, AT) and temperature (two levels: 10 °C, 14 °C) on RGR ($n=5$). The assumption of normality and homoscedasticity were tested as for previous analyses. Tukey's HSD *post-hoc* test was used to examine pairwise significant differences between the factor combinations. All analyses were performed in R version 4.2.2 (R Development Core Team 2022) using the packages "*GAD*" (Sandrini-Neto and Camargo 2022), "*vegan*" (Oksanen et al. 2022).

Fits for the quenching parameters versus the E/E_k ratio were compared between treatments including the initial control (T0) by means of Extra-sum of squares *F*-test in GraphPad Prism (GraphPad Software Inc.). Since time and temperature had no significant effect on these parameters, the data were pooled among Ti × Te combinations.

Results

Growth and fertility

RGR was significantly affected by the culture medium ($P=0.019$), although with slight differences, but not by temperature ($P=0.059$) nor by their interaction ($P=0.209$) (Table S2). The highest values were at 14 °C in AT (0.027 ± 0.004 day^{-1}) and the lowest at 10 °C in SW (0.013 day$^{-1}\pm0.001$ SE) (Fig. 3).

The receptacles began to develop at T2 (Fig. 4a, b). The fertile conceptacles released viable zygotes (Fig. 4b, c, d). After two weeks of cultivation (T4), the mean number was 444.6 ± 182.8 germlings Petri^{-1} and at T5 it was 45.3 ± 6.5 germlings Petri^{-1} (Table S3).

Photosynthetic performance

At T1, T2 and T3, F_v/F_m values showed a significant interaction between culture medium and temperature (Table S4, $F_{(1,36)}=23.21$, $P<0.001$), i.e. slight but significant differences between media changed with temperature. At 10 °C, the values between the two media were comparable, while 14 °C AT (0.71 ± 0.01) had higher values than SW (0.65 ± 0.04) (Fig. 5a). The F_v/F_m values under the AT treatment were similar to those of the control at T0 (0.73 ± 0.02), they decreased by 11% in the SW treatment at 14 ºC after three weeks (Two tailed *t*-test, $t=3.82$, df = 13, $P=0.002$).

The interaction between temperature, time and medium had a significant effect on F_0 (Table S5), therefore the differences changed over time depending on temperature and culture medium. Specifically, at AT, F_0 was stable from T1 to T3 at both temperatures, while at SW, F_0 decreased significantly by 17% at 10 °C, but increased by 23% at 14 °C (Table S5).

Analysis of the $rETR_{max}$ values derived from the RLCs curves revealed a significant Ti × Me interaction, indicating that the temporal patterns of $rETR_{max}$ values differed significantly between media (Table S6). Specifically, $rETR_{max}$ values increased over time in the AT samples, from 95.12 ± 9.21 (T1) to 153.06 ± 32.75 (T3) at 10 °C and from 93.12 ± 13.58 (T1) to 128.83 ± 9.69 (T3) at 14 °C, while they remained stable in SW, with a mean pooled value of 57.4 ± 15.1 (Fig. 5b). Respect to initial control values (T0), $rETR_{max}$ increased by twofold between T0 and T3, while thalli grown in SW had a

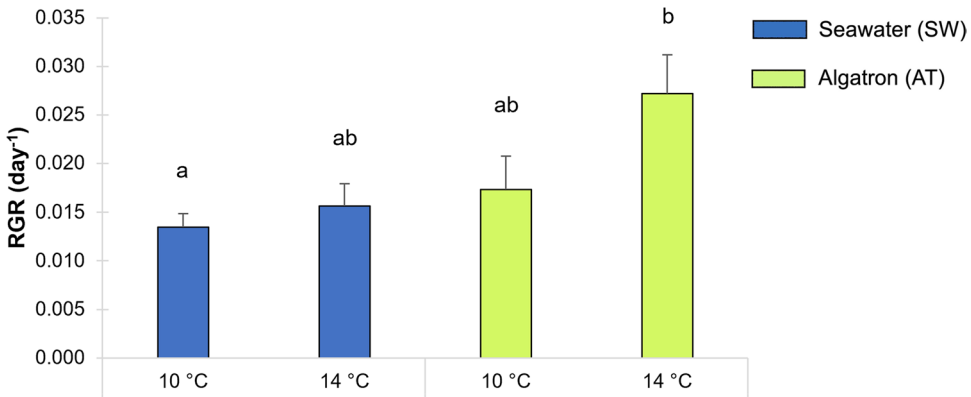

Fig. 3 Relative growth rate (RGR) of fronds of *Gongolaria barbata* in the two culture media (SW, AT) and temperatures (10 °C, 14 °C). Data are mean ($n=5$) ± SE. Different letters above the bars indicate significant differences at $P<0.05$ due to the treatment according to Tukey's HSD *post-hoc*

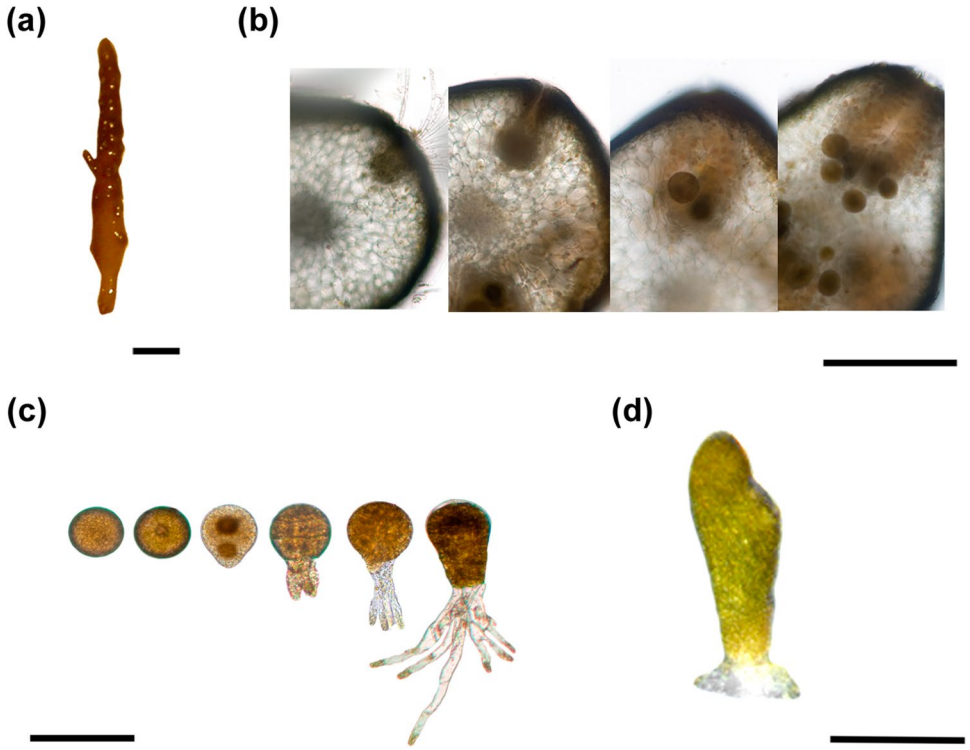

Fig. 4 Development and maturation *of Gongolaria barbata* receptacles (14 °C in AT); **a)** apical receptacle (scale bar = 2 mm); **b)** development *of G. barbata* conceptacles (scale bar = 500 µm) **c)** fertilization and development *of G. barbata* embryos during 1 week (scale bar = 200 µm); **d)** two-week-old germlings (scale bar = 1 mm)

significant effect of medium, regardless of temperature and time, for αETR values (Fig. 5c): AT samples showed higher values (0.69 ± 0.07 at 10 °C; 0.66 ± 0.06 at 14 °C) than SW (0.57 ± 0.10 at 10 °C; 0.52 ± 0.13 at 14 °C).

Maximum non-photochemical quenching (NPQ_{max}) showed significant differences between the different culture media, independent of the temperature and time (Fig. 5d): samples grown in SW showed slightly higher values (4.09 ± 0.42 at 10 °C; 3.84 ± 0.33 at 14 °C) compared to samples grown in AT (3.58 ± 0.50 at 10 °C; 3.22 ± 0.64 at 14 °C).

Model comparison from pooled q_N and q_P data (Ti × Te) revealed significantly different responses curves among culture media and with control values (T0) (Fig. 6, Table S7). Differences in photochemical quenching (q_P) were mostly attributed to changes in E/E_{k50} (i.e. the E/E_k value at which half of the reaction centers were open, T0 < AT < SW) and the HillSlope (i.e. the rate at which q_P declined, SW < T0 < AT). At the light-limiting region, photochemical quenching (q_P) was close to 1 for all treatments (Fig. 6a). At $E/E_k = 1$, q_P values from the control and AT treatment were around 0.7–0.9, indicating that around 80% of reaction centers were opened. In contrast, thalli of *G. barbata* cultured with SW had lower q_P values with a greater variability (ranging from 0.5 to 0.75) and 50% of reaction centers were still open at higher E/E_k values than for the control or AT (Fig. 6a). Within the RLC light saturating region ($E/E_k > 1$), SW-cultured thalli showed the lowest values (Fig. 6a).

Non-photochemical quenching (q_N) values began rising exponentially at lower E/E_k values in AT than in SW or T0. Only T3 had a significant effect on q_N of *G. barbata*, with higher values at 10 ºC than 14 ºC (Extra-sum of squares *F*-test, AT: $F_{(4,40)} = 3.57$, $P = 0.014$; SW, $F_{(4,40)} = 14.2$, $P < 0.0001$). For SW the greatest differences were detected around the theoretical optimal range for photosynthesis ($E/E_k = 1$, Table S4). The differences in the q_N curves were mainly due to the E/E_{k50} values. Although half of the maximum q_N was reached at $E/E_k \sim 0.6$ for both culture media, the initial control samples required higher saturation to reach 50% of the maximum q_N capacity than thalli cultured in SW or AT (Table S7, Fig. 6b). Nevertheless, SW and T0 had a similar rise in q_N towards the RLC light saturating region, whereas thalli from AT experienced a steadier rise in q_N, based on their lower HillSlope values (Fig. 6b, Table S6).

Discussion

The RGR of *Gongolaria barbata* in AlgatronCifo® (AT) was higher than in seawater, probably due to the presence of phytohormones and the polysaccharide alginate, which have been shown to promote growth (Briceño-Domínguez et al. 2014; Sujeeth et al. 2022; Umanzor et al. 2022). The highest growth was observed at 14 °C, which corresponds to the spring temperature in the Gulf of Trieste, when vegetative development of *G. barbata* is known to be maximal (Falace et al. 2006) and coincides with the temperature at which Orfanidis (1991) found the highest growth for the same species.

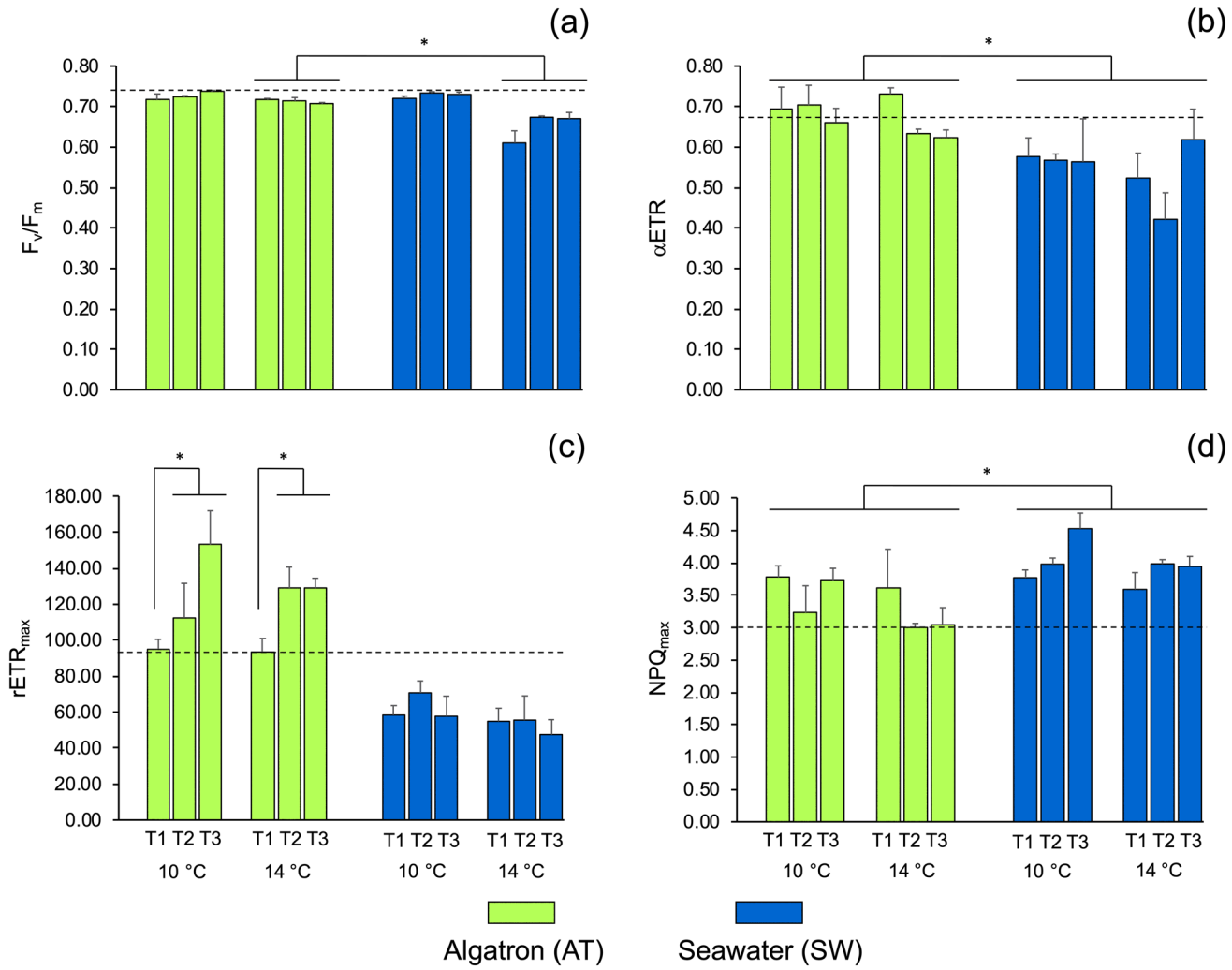

Fig. 5 F_v/F_m, α_{ETR}, $rETR_{max}$, and NPQ_{max} of *Gongolaria barbata* thalli at the three times (T1 = 7 days, T2 = 14 days, T3 = 21 days) and temperatures (10 °C and 14 °C) of exposure to the two media, namely Algatron (light green bars) and seawater (blue bars). Dotted lines indicate values at T0. Units for F_v/F_m and NPQ_{max}: dimensionless; $rETR_{max}$, µmol electrons m^{-2} s^{-1}; αETR, mol electrons (mol photons)$^{-1}$. Data are mean ($n = 3$) ± SE

Fertile receptacles also developed exclusively at 14 °C-AT, followed by the release of viable zygotes. The number of zygotes released, and the survival of embryos was comparable to previous *ex-situ* cultures from receptacles harvested in the same geographical area (Savonitto et al. 2021). Therefore, this study indicates that the switch from vegetative to reproductive phase can be initiated by AT within two weeks. In contrast, thalli of *G. barbata* cultured for 16 months at different temperatures in von Stosch's enriched SW (i.e., from -1 to 33 °C) remained sterile under all conditions (Orfanidis 1991). In the case of *Ericaria barbatula* (Kützing) Molinari & Guiry, which was cultivated in SW, fertility was only achieved after six months (Papadimitriou et al. 2022). The biostimulant AT has been shown to significantly increase plant growth and flowering of *Lobivia* spp. (Prisa 2021). An increase in flowering has been documented in plant crops treated with macroalgal biostimulants (Ali et al. 2021), which has been linked to the phytohormones (or phyco-elicitors), especially cytokinins, contained in the extracts (Khan et al. 2009).

Our study also showed that *G. barbata* cultured with a commercial algal biostimulant AT increased its photosynthetic capacity ($rETR_{max}$) and efficiency (α_{ETR}) by 2 to 2.5-fold and enhanced photochemical quenching (q_P) compared to its culture in seawater (SW). On the one hand, the higher photosynthetic efficiency (α_{ETR}) in AT could be related to an increase in the biosynthesis of light-harvesting centers due to N enrichment. A higher density of PSII centers was observed in the kelp *Saccharina latissimi* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders grown under optimal N supply and temperature (Gerard 2008). Nitrogen has been shown to improve the physiological performance

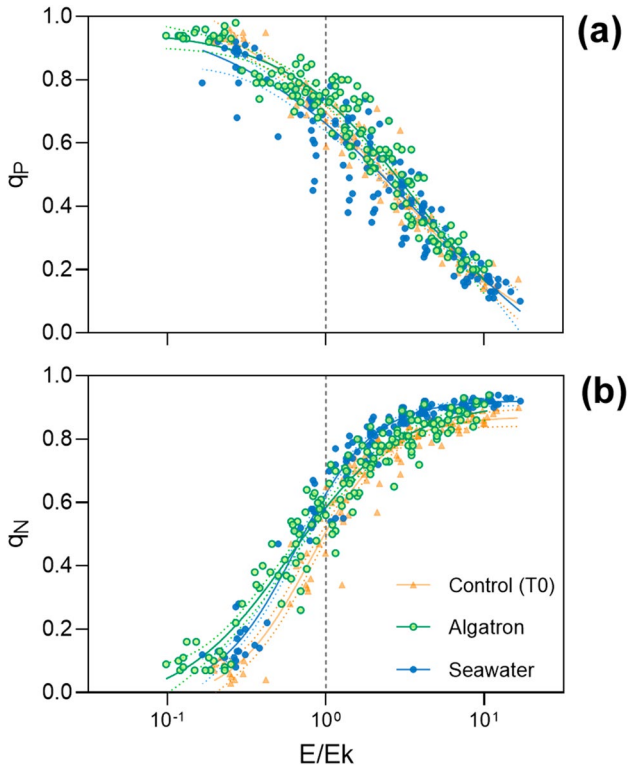

Fig. 6 **a)** Photochemical (q_P) and **b)** non-photochemical (q_N) quenching of thalli of *Gongolaria barbata* exposed to two culture media, namely Algatron (green dots) and seawater (blue dots) and the initial control (T0, orange dots), represented against the ratio between incident irradiance (PAR = E) and the saturating irradiance for photosynthesis derived from the rapid light curves (E_k), as E/E_k. Data from different experimental times (T1, T2, T3) and temperatures (10, 14 °C) were pooled with a total of $n = 144$ per culture media and $n = 95$ for T0

and growth rates of fucoid algae and mitigate the negative effects of stress (Fernández et al. 2020; Gerdol et al. 2020). Apart from the higher nutrient availability, the addition of the biostimulant may have induced changes in algal metabolism and signaling pathways specifically related to nutrient uptake and/or nutrient translocation, a mechanism that has also been observed in plants where biostimulants improved nutrient use efficiency (Jannin et al. 2013; Saa et al. 2015; Sujeeth et al. 2022).

Molybdenum was one of the micronutrients in AT (Table S1), naturally occurring in *M. pyrifera* (Iparraguirre et al. 2023), that plays a role in nitrate reduction and ion absorption in seaweeds (DeBoer 1981). This micronutrient is known to promote leaf production and fruiting in plants in synergy with other biostimulant components (La Bella et al. 2021). For *G. barbata*, Ak et al. (2020) reported that Mo concentrations were detected in field samples, but its concentration increased eightfold when cultured with F/2 medium (0.03 µM $Na_2MoO_4.2H_2O$, Guillard 1975). The Mo concentrations of the biostimulant used in our experiment were 0.04–0.05 µM, which added to the average Mo concentration in seawater (0.10 µM, Abbott 1977) would result in a 1.5-fold enrichment that can be assimilated by *G. barbata*. In this context, it can be hypothesized that bio-enrichment with Mo may have promoted the photophysiology of *G. barbata*, as is the case with other micronutrients such as Cu (Celis-Plá et al. 2018).

Non-photochemical quenching parameters (NPQ_{max}, q_N) followed an opposite trend to the other photo-physiological parameters, with higher values in SW treatments. NPQ values represent the ability to efficiently dissipate excess energy through non-photochemical quenching, indicating a high photoprotective capacity (Celis-Plá et al. 2014). High NPQ_{max} values may indicate environmental stress in response to high light intensity, desiccation, temperature, or nutrient deficiency (Ballottari et al. 2007; Gerotto et al. 2011; Cardol and Krieger-Liszkay 2017). In our experiment, culture media were frequently renewed (every 2–3 days) and provided at similar (SW) or higher concentrations than in their natural habitat (i.e., AT had a 58-fold higher DIN). However, a possible N-limitation cannot be ruled out, as the turnover rates in the field may provide a higher supply in the field than under laboratory conditions. This fact would explain why the physiological performance of SW fronds and the initial control were more similar (T0), while the photosynthetic responses in AT were promoted even above control values. On the other hand, nutrient availability and ratios can interact with their uptake and assimilation and affect photosynthesis and growth (Roleda and Hurd 2019). According to the DIN:SRP ratio at which nutrients were supplied in each treatment (121 and 382 for SW and AT, respectively), there would be an external P-limitation in both culture media. According to Dodds (2003), the DIN:SRP ratio should be interpreted altogether with the absolute amounts of each nutrient to avoid misuse. In this regard, although AT would indicate a greater P-limitation (due to 58 × N supply), PO_4^{3-} values in AT were twice those in SW (Table 1), and therefore SW would be more P-limited. Under nutrient limiting situations, and particularly P, the photochemical pathway of ATP synthesis is limited leading to lower ETR values (Geider et al. 1993; Wykoff et al. 1998). Such disruptions in ATP synthase leads to marked proton gradients (ΔpH) across thylakoidal membranes (Muller et al. 2001), due to lower proton requirements and ATP consumption (Gauthier and Turpin 1997). These alterations have been linked to an enhanced energy dissipation via non-photochemical pathways (Krieger et al. 1992), detected in nutrient limited photoautotrophs by increased NPQ or q_N (e.g. Dal Bosco et al. 2004; Rodríguez-Román and Iglesias-Prieto 2005; Weng et al. 2008; Zhang et al. 2019). This hypothesis would be supported by the lower q_P values and enhanced non-photochemical response (q_N, NPQ_{max}) we found in *G. barbata* cultured with SW, a culture medium where it was particularly

evident in the saturating region of the RLCs due to a greater need of dissipating excess heat.

Long-term series of PO_4^{3-} and DIN concentrations in the Gulf of Trieste have shown that the availability of key nutrients in these coastal waters has changed, leading to almost permanent P deficiency over the last two decades (Cozzi et al. 2020). Increasingly dystrophic waters, together with climate change, are likely responsible for the major ecosystem shifts in the Gulf of Trieste, where habitat-forming macroalgae have almost completely disappeared and been replaced by ephemeral species (Orlando-Bonaca and Rotter 2018). Thus, higher nutrient concentrations in coastal areas bordering lagoons and freshwater discharges could be an explanation for the greater presence of *G. barbata* populations in these areas.

The enhancing effect of the *Macrocystis* extract occurred only during the first week of treatment, which probably contributed to the overall higher growth and enhanced physiological condition of the treated thalli and could be due to the presence of mannitol, which serves as an energy reserve in the short term (Briceño-Domínguez et al. 2014; Hurd et al. 2014). The nutritional strategy of *G. barbata* is largely unknown, but the higher photosynthetic and growth capacity when cultured with AT suggests that *G. barbata* may benefit from transient pulses of high nutrient concentrations, as previously observed in *Cystoseira humilis* Schousboe ex Kützing (Vaz-Pinto et al. 2014). The ability to surge uptake could be particularly beneficial for *G. barbata* populations growing in a coastal area such as Grado, where nutrient fluctuations and transient nutrient surpluses occur during the largest freshwater flows (Acquavita et al. 2015; Cozzi et al. 2020). Future studies should address the nutrient uptake, assimilation, and storage abilities of this species to fully understand its nutritional physiology.

Temperature had a secondary but significant role in the photo-physiological responses of *G. barbata*, interacting mainly with the culture media. This interaction could be due to higher nutrient demand through increased photosynthesis and growth rate at 14 ºC, as shown by the higher effective quantum yield of PSII (F_v/F_m), photosynthetic capacity ($rETR_{max}$) and greater RGR with algal biostimulant treatment (AT). At low temperature, photosynthetic electron transport may be limited by the increased stiffness of the thylakoid membrane and the resulting reduction in the movement of the proteins of the photosynthetic apparatus across the membranes. Moreover, the enzymatic reactions of the Calvin Benson cycle operate at reduced rates at low temperatures and consume less ATP and NADPH. These conditions generate an excess of light energy that can be dissipated by boosting non-photochemical pathways. However, this mechanism would only explain the higher non-photochemical quenching of thalli grown at 10 ºC in SW at T3, which is probably related to the greater P-limitation experienced after three weeks under that condition.

We cannot exclude that other unknown hormone-like compounds of the commercial biostimulant contributed to the improvement of the overall physiological state as well as the growth and reproduction of *G. barbata*. The components of the biostimulants are thought to act synergistically (Fornes et al. 2002; Vernieri et al. 2005; Yakhin et al. 2017; La Bella et al. 2021), as the uptake of the whole extracts is more beneficial than that of their individual components (Ali et al. 2021). Furthermore, the molecular mechanisms of action of these extracts are not yet fully understood (El Boukhari et al. 2020; Hurtado and Critchley 2020; Sujeeth et al. 2022) and require further functional studies to determine how they work and what effects they have on nutrient uptake and algal growth. Further investigations are required to test dose–response, in conjunction with nutrient-enriched media, to promote thalli and seedling growth. The mechanisms of action of these seaweed-derived biostimulants, using -omics techniques (e.g. metabolomics and/or transcriptomics) will help to unravel the effects of the various components.

Macroalgal-derived biostimulants offer an innovative strategy to improve macroalgal culture by boosting growth and promoting early fertility for sustainable restoration without harming the natural stands. Triggering reproduction under controlled laboratory conditions could ensure the availability of seedlings for a longer periods, thus promoting the restoration of *Cystoseira s.l.* forests on a larger scale, regardless of the availability of donor populations.

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Author contributions SK, RSdP, EBE, AF conceived the study; SK, GS performed the lab measurements; SK, RSdP, EBE, GS, SB, SN, AA, AF performed data analysis; SK, RSdP, EBE, AF wrote the original draft of the manuscript. All authors contributed to the revision of the manuscript and approved the submitted version.

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Data availability The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Declarations

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

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